CELLULASE PRODUCTION BY *Rhizobium*

BY

VICTOR MANUEL MORALES

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1981

I dedicate this work

to my children

Susana and Victor.

IM19-82-0027

ACKNOWLEDGEMENTS

The author would like to express his deep appreciation to the chairman of his committee, Dr. David H. Hubbell for his constant support, understanding and guidance. He would like to thank his committee members Dr. Chesley B. Hall, Dr. James F. Preston, Dr. Bob G. Volk and Dr. Edward M. Hoffmann for their assistance and suggestions.

He would also like to thank Mr. Manuel Mesa and Mrs. Louise Munro for their technical assistance and Garnet Jex, Frederick Schipul, Anne Barkdoll, Ana Moliner and Karen Kanudtsen for their friendship.

The author thanks the scientists listed in Table 1 who provided the Rhizobium cultures used in this study.

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CELLULASE PRODUCTION BY *Rhizobium*

By

Victor Manuel Morales

December 1981

Chairman: Dr. David H. Hubbell
Major Department: Soil Science

The production of cellulase by *Rhizobium* species was studied. *Rhizobium trifolii* cellulase is induced by a variety of polysaccharides, including celluloses and hemicelluloses. Cellobiose and myo-inositol also induce enzyme production but mannitol represses it at concentrations higher than 0.25%.

Cellulase production by "slow-growing, cowpea type" rhizobia is more stringently regulated. Few substances induced the production of cellulase in the strains studied of this group of *Rhizobium*. Effective inducers were carboxymethyl-cellulose, gulconate and myo-inositol.

Plant root substances were shown to stimulate cellulase production by *Rhizobium*. Root substances soluble in phosphate citric acid buffer stimulated cellulase production as measured by the viscosimetric assay, while root solids increased the enzymatic activity as determined by the release of reducing

groups. This is an indication of a differential stimulation of enzyme production by the plant root.

Cellulase production was very low under all conditions tested. Most of the enzyme is loosely bound to the capsular material and/or the cell surface. The enzyme in fast-growers is an 1,4-β-D-glucan-4-glucanohydrolase (endo-glucanase EC 3.2.1.4) with specificity for high molecular weight oligosaccharides. In slow-growers enzyme extracts show small decreases of viscosity of carboxymethylcellulose solution with a relatively large release of reducing groups.

Rhizobium cellulase loses activity under low ionic strength conditions. Carbohydrate also seems to help stabilize the enzyme in raw extracts. The proteins, after separation from the carbohydrates, are difficult to redissolve. Solubilization is achieved when glycine, urea and small amounts of calcium and magnesium are present in the solution.

There was no correlation between infectiveness of Rhizobium trifolii strains and cellulase production. One strain, which lacks the nodulation plasmid, proved to be capable of producing cellulase at the same rate as its parental infective strain.

INTRODUCTION

The infection of legume roots by Rhizobium is a complex process which shows a high degree of specificity. The expression of this specificity occurs prior to the initiation of the infection thread (Li and Hubbell, 1969). The binding of the bacterium to the root hair surface through a lectin bridge has been reported to play a role in the specificity of infection (Dazzo et al., 1976). However, the biochemical mechanism by which the infection thread is initiated is unknown. Cell wall-degrading enzymes are likely to be involved since they are important in cell wall-growth processes. Rhizobium produces several polysaccharide degrading enzymes, notably pectinases (Hubbell et al., 1978), hemicellulases and cellulases (Martinez-Molina et al., 1979) when growing in artificial media. The purpose of this research was to define the conditions which induce the production of cellulases by Rhizobium, to purify and characterize the enzymes, and to evaluate their possible involvement in the infection process.

LITERATURE REVIEW

The Rhizobium-legume association is the symbiosis with highest importance for agriculture. The bacteria, Rhizobium, induce nodule formation in the roots of leguminous plants. Inside these nodules the microbe fixes atmospheric nitrogen (N_2) to ammonia which is then used by the plant. Agronomic exploitation of this plant-bacteria symbiosis is of great importance for agriculture. It has been calculated that the use of leguminous plants in agriculture provides an annual input of fixed N approximately equal to that fixed industrially for fertilizer (Delwiche, 1978).

Rhizobia are true bacteria belonging to the family Rhizobiaceae; they are gram negative rods which can live saprophytically in the soil and the rhizosphere of leguminous and nonleguminous plants (Vincent, 1974). They are divided into two main groups based on their growth rate on yeast extract mannitol agar medium (YEMA). The fast-growers generally nodulate temperate legumes and the slow-growers usually nodulate tropical legumes. The difference in growth rate reflects true biochemical and genetic differences (Graham, 1975; Martinez-de Drets and Arias, 1972).

One important characteristic of the establishment of the symbiosis is its specificity. Certain strains of rhizobia can

nodulate only certain species of leguminous plants. Based on this, rhizobia have been classified in groups according to which plants they can nodulate. This classification, although useful for practical work, has been criticized because of its lack of a biochemical and genetical basis and because of promiscuous behavior of some strains (Graham, 1975).

The specificity of nodulation implies the existence of a mechanism by which a mutual recognition of host and symbiont occurs (Dazzo and Hubbell, 1981). In the case of a homologous (nodulating) combination of plant and microbe, a complex infection process occurs. Knowledge of this process of infection of legume roots has been recently reviewed (Bauer, 1981; Bhuvaneswari, 1981; Dart, 1974, 1975, 1977; Dazzo and Hubbell, 1981; Solheim and Paxton, 1981).

Fast-growing Rhizobium, when proliferating in the rhizosphere of a compatible host, induces the deformation of root hair. "Branched," "moderately curled" and "markedly curled" deformations have been defined as distinct categories. "Markedly curled" root hairs have a characteristically twisted tip known as the shepherd's crook. "Markedly curled" root hairs appear only in homologous combinations (Yao and Vincent, 1976).

Rhizobia enclosed in the "shepherd's crook" trigger the development of a tubular structure of plant origin called the infection thread. The infection thread enveloping the bacteria grows toward the base of the root hair and eventually, passing from cell to cell, penetrates the root cortex. Rhizobia are

released from the infection thread into the cytoplasm of certain cortical cells. The bacteria are enveloped by plant plasmamembrane, forming what is called "bacteriodal vesicles." In these vesicles, rhizobia are transformed into bacteroids which are responsible for N_2-fixation in the nodule (Basset et al., 1977; Paau et al., 1978; Robertson et al., 1978).

The recognition between host and microsymbiont occurs before the initiation of the infection thread (Li and Hubbell, 1969). The most promising current hypothesis to explain the specificity of infection is that host plant lectins interact specifically with microbial cell surface carbohydrates attaching the bacteria to the root hair surface (Dazzo et al., 1976). In a more complete model, proposed by Graham (1981), the attachment is one of the steps involved in recognition but not the only one. Following attachment, there is a signal production and reception sequence which induces responses by the symbionts and triggers the infection thread formation. The nature of these signals is unknown, although cell surface polysaccharides are implicated (Yao and Vincent, 1976).

Ljunggren and Fahraeus (1961) proposed that rhizobia induce the production of polygalacturonases by the plant. These enzymes would "soften" the root hair cell wall allowing the Rhizobium to penetrate the plant cell and then trigger the infection thread formation. The involvement of polygalacturonases in the infection process has been under discussion with published results against (Lillich and Elkan, 1968; McMillan and Cooke, 1969; Solheim and Raa, 1971) and favoring

(Palomares, 1975; Olivares et al., 1977; Verma and Zogbi, 1978). Confirmation of the Ljunggren and Fahraeus hypothesis must contend with the low levels of enzyme activity and the limits of reliability of the assays.

It has been found, recently, that rhizobia not only produce small amounts of polygalacturonases (Hubbell et al., 1978), but also produce cellulases and hemicellulases (Martinez-Molina et al., 1979).

The involvement of polysaccharide-degrading enzymes in the infection process is also suggested by electron microscope data (Callaham, 1979; Chandler, 1978; Napoli and Hubbell, 1975). Rhizobia enclosed in the shepherd's crook multiply and locally disrupt the cell wall growth. A layer of new material is then deposited at the infection site. This is apparently similar to the defense mechanism observed at the point of entry of microbial pathogens (Aist, 1977). Cell wall-degrading enzymes are implicated as elicitors of defense responses in plants (Hahn et al., 1980; Lyon and Albersheim, 1980). However, the mechanism for infection thread formation must differ since callose, the material usually deposited at the site of cell wall injury, is not observed at the infection thread initiation point (Callaham, 1979). The type and quantity of polysaccharide-degrading enzymes may be this difference. Plant polysaccharide-degrading enzymes are thought to play a role in cell wall-growth processes (Roland and Vian, 1979) and Rhizobium enzymes may only modify the regulation of the cell wall-growth.

There have been many recent advances in the genetics of Rhizobium in general and with respect to nodulation (Beringer, 1980, Beringer et al., 1980). Fast-growing Rhizobium strains possess several large plasmids which may account for up to 10% of the total genetic information (Casse et al., 1976; Prakash et al., 1980). It has been established that the genes necessary for nodulation reside in a plasmid (Zurkowski and Lorkiewicz, 1979). This nodulation plasmid has been denominated sym plasmid. Transfer of this sym plasmid to a related microbe, such as Agrobacterium tumefasciens, makes it capable of forming root nodules in the proper host (Hooykaas et al., 1981).

Polysaccharide-degrading enzymes have been considered to play an important role in many plant diseases (Bateman, 1964, 1976; Bateman and Millar, 1966; Byrde, 1979). Most phytopathogenic microorganisms are capable of producing polysaccharide-degrading enzymes; this is a feature plant pathogens share with members of the saprophytic flora and fauna of the soil. It is thus apparent that the ability to produce polysaccharide-degrading enzymes does not in itself enable a microorganism to be a plant pathogen, but it may be an essential feature for the pathogenic capabilities of certain pathogens (Bateman, 1976).

Cellulases are produced by bacteria, fungi, actinomycetes, protozoa, insects and molluscs (Coughland and Folan, 1979; Gooday, 1979). Not all cellulolytic microbes can degrade crystalline cellulose but all of them can hydrolyse amorphous or substituted celluloses. Reese et al. (1950) proposed two

types of cellulases, C_1 and C_x. The enzymes designated C_1 were supposed to be nonhydrolytic, but caused disaggregation of the cellulose chains of the native material, thereby allowing the hydrolysis of the products by the enzymes designated C_x. The C_x enzymes hydrolyzed the chains made accessible by the C_1 cellulases. More recently, highly purified C_1 material has been isolated and demonstrated to be an exo-β-1,4 glucanase (Nisizawa et al., 1972; Shikata and Nisizawa, 1975). Thus, hydrolysis of native cellulose is thought to result from synergistic action of endo- and exo-glucanases, which form multienzyme complexes on the surface of substrate molecules (Coughlan and Folan, 1979).

The enzyme components of a cellulase complex are: i) 1,4-β-D-glucan 4-glucanohydrolases (endo-1,4-β-D-glucanases; EC 3.2.1.4), which preferentially hydrolyse internal glycosidic linkages of cellulosic substrates; ii) 1,4-β-D-glucan cellobiohydrolases (exo-cellobiohydrolases; EC 3.2.1.91), which preferentially cleave cellobiose units from the nonreducing end of cellulose chains; and iii) β-glucosidases (cellobiase; EC 3.2.1.21), which cleave cellobiose units produced by the endo- and exo-glucanases to give glucose (Coghlan and Folan, 1979).

The two most commonly used assays for C_x cellulases are the measurement of the production of terminal reducing groups or of glucose and the measurement of the decrease in viscosity of a solution of carboxymethylcellulose (CMC). The viscosimetric method is much more sensitive for endo-glucanases, since a random break in the cellulose chain may reduce

viscosity by 50% but causes almost no increase in reducing groups (Ryu and Mandels, 1980).

Cellulase is an inducible enzyme system in most microbial systems studied. Cellulose seems to be the best inducer for the complete cellulase complex (Coughlan and Folan, 1979). Other inducers are sophorose (Mandels et al., 1962) (2-O-β-D-glucopyranosyl-α-D-glucose) and lactose (Ryu and Mandels, 1980). Cellulase biosynthesis is controlled by both catabolite repression and by end-product inhibition (Montenecourt et al., 1979; Reese, 1977).

The components of the cellulase system vary according to the microorganism and the substrate on which it grows. The number of enzymatic components for C_x enzymes range from 1 to 12 and for the C_1 group from 1 to 3. They can be glycoproteins, in which case the size of the oligosaccharide is another source of variation. Endoglucanases differ in the degree of randomness of the splitting of glycosidic linkages and in the limiting size of the oligosaccharide on which they can act. Some have no activity on substrates smaller than 5 units (Reese, 1977). Many cellulases are able to catalyze the hydrolysis of xylan (Capon and Thompson, 1979). Urbanek et al. (1978) described a purified enzyme from Phoma hibernica capable of hydrolysis of β-1,4 glycosidic linkages formed by different hexoses. It can degrade xylan, galactomannan, glucomannan and galactoglucomannan.

Rhizobium trifolii strains are capable of synthesizing cellulose (Napoli et al., 1975), small molecular weight β-2

linked glucans (York et al., 1980; Zevenhuizen and Scholten-Kaerselman, 1979) and acidic heteropolysaccharides containing galactose, glucose, glucuronic acid and pyruvate (Robertson et al., 1981). The structure of these heteropolysaccharides are complex with branches and different types of linkages such as α-1,4; β-1,4; β-1,6; β-1,3 (Robertson et al., 1981; Somme, 1974, 1980).

The role of microbial polysaccharides in nature is not clear. They are thought to play a role in survival to desiccation, food storage, protection against viruses, clumping and flocculation and adhesion to surfaces (Deinema and Zevenhuizen, 1971; Harris and Mitchell, 1973). The latter role seems to be very important in pathogenesis and the establishment of symbiotic relationships such as Rhizobium-legume root nodules (Dazzo and Hubbell, 1981).

Rhizobium seem to be unable to hydrolyze their own extracellular polysaccharide but the production of phage-induced extracellular-polysaccharide depolymerases (Higashi and Abe, 1978).

MATERIALS AND METHODS

The strains of Rhizobium used in the study, their sources and legume host are listed in Table 1.

The Rhizobium strains were kept on yeast extract mannitol agar (YEMA) slants and/or in lyophilized form.

The infectivity of the strains was defined as the ability to induce the formation of nodules in the roots of host plants growing in Gibson's partially enclosed seedling assemblies (Vincent, 1970).

Inocula for experiments were prepared by resuspending the bacterial growth from petri plate cultures in sterile deionized water. The bacterial population was counted using a Petroff-Hauser chamber and the amount of the bacterial suspension to be used as inoculum calculated to obtain an initial population of 1.5×10^7 cells/ml in the culture.

Cellulase Activity

Three methods were used for assaying enzymatic activity. The plate-cup method as described by Dingle et al. (1953) using carboxymethylcellulose (hereafter called CMC; type 7HF, Hercules Inc., Wilmington, DE) in phosphate-citric acid buffer (PCA buffer; 0.1M K_2HPO_4 and 0.1M citric acid mixed to pH 5.2) as substrate and agar as solidifying agent. The plates were

TABLE 1: Sources and characteristics of *Rhizobium* strains used in the research.

Species	Strain denomination	Host	Infectiveness	Source
Rhizobium trifolii	BAL	*Trifolium repens*	+	F. B. Dazzo
"	BART A	"	-	G. Fahraeus
"	0403	"	+	"
"	0435	"	+	A. N. McGregor
"	0435-2	"	-	"
"	521	"	+	W. Zurkowski
"	521nod$^-$8	"	-	"
Rhizobium sp. "cowpea type"	CIAT 79	Wide range*	+	P. H. Graham
"	CB82	*Stylosanthes* sp.	+	R. A. Date
"	CB1650	"	+	"
Rhizobium japonicum	J61	*Glycine max*	+	U. of Florida collection**

*Infectivity tested on *Macroptilium atropurpureum*. Same as CB756.

**Original source unknown.

incubated for 48 hours at 35°C and developed by flooding with a 1 w/v solution of hexadecyltrimethylammonium bromide (Eastman Kodak Co., Rochester, NY). The enzymatic activity was proportional to the diameter of the hydrolysis zone.

Cellulase activity was also measured by the decrease in viscosity of a solution of CMC (0.2% w/v) as described by Martinez-Molina et al. (1979) using Cannon-Fenske viscometers.

The third method used was the measurement of the increase in reducing power at 30°C of a reaction mixture consisting of 1 ml of CMC solution (0.2% w/v) in PCA buffer and 1 ml of enzyme extract. Reducing sugars were assayed by the method of Somogyi-Nelson (Somogyi, 1952).

Enzymatic activity was expressed as enzyme units (EU) per 100 mg of dry weight of bacteria or as EU per ml of enzyme extract. Results of the viscosimetric assay were expressed as EU (see Appendix I) or as percentage of decrease of viscosity after 24 hours of incubation.

One enzyme unit (EU) was defined as the amount of enzyme that reduces the viscosity of CMC solution (0.2% w/v) by 50% in 10 hours or has a slope (plot of the reciprocal viscosity against reaction time, hereafter called slope $1/\eta_s$) of 1.883×10^{-2} $hour^{-1}$ or produces an increase in reducing power in a CMC solution equivalent to 25 µg of glucose in 24 hours.

Enzymatic activity was corrected for microbial growth as explained in Appendix II.

Glucose was measured by the glucose oxidase method (Sigma Chemical, St. Louis, MO).

Screening for Culture Conditions Suitable for Cellulase Production

To screen for a medium in which cellulase production is induced, three strains of Rhizobium (R. trifolii BAL, R. japonicum J61 and Rhizobium sp. CIAT 79) were grown in two different basal media, Bergersen's (Bergersen, 1961) and RDM (Ronson and Primrose, 1979). Six different carbon sources were tested with each basal medium: myo-inositol (0.4% w/v), arabinose (0.5% w/v), potassium gluconate (0.5% w/v), sodium succinate (0.5% w/v), sodium malate (0.5% w/v) and xylose (0.5% w/v; all obtained from Sigma Chemical, St. Louis, MO). Media were dispensed in screw cap test tubes in 10 ml amounts and, after inoculation, incubated in an orbital shaker at 28°C and 120 rpm. After 8 days of incubation, the cultures were sonicated for 1 min at 60W (Model W140; Heat Systems-Ultrasonics Inc., Plainview, NJ) and centrifugated at 15,300 x g for 15 min at 4°C. Cellulase activity of the supernatants was assayed by the plate-cup method.

Effect of Different Concentrations of Myo-inositol or Potassium Gluconate on Cellulase Activity

In order to investigate the effect of the concentration of myo-inositol and gluconate on cellulase production, two strains of Rhizobium (BAL and CIAT 79) were grown in Bergersen's broth with different concentrations of these two C sources (0.25, 0.5, 1.0, 1.5 and 2.0% w/v). The media were dispensed in flasks in amounts of 100 ml, and inoculated and incubated as previously described. After 8 days of incubation, the cells were precipitated by centrifugation at 3,550

x g for 1 hour at 4°C. The pellet from each 100 ml of medium was resuspended in 10 ml of PCA buffer and sonicated for 1 min at 60W. The broken cells were precipitated by centrifugation at 15,300 x g for 15 min at 4°C. The supernatant (hereafter called EF enzyme extract) was tested for cellulase activity by measuring the release of reducing groups.

Induction of Cellulase Production by Different Carbohydrates

Rhizobium trifolii strain BAL and Rhizobium sp. "cowpea group" strain CIAT 79 were grown in Bergersen's medium with 0.1% w/v mannitol and with 5 g/l of the following substances: CMC, xylan, locust bean gum, gum arabic and cellobiose (all obtained from Sigma Chemical Co., St. Louis, MO, with the exception of CMC). Three nitrogen sources were used: NH_4Cl (0.1% w/v), KNO_3 (0.1% w/v) and sodium glutamate (0.11% w/v). The media were inoculated and incubated as previously described. After 8 days the cultures were harvested and EF enzyme extracts prepared as described except that the cells were resuspended in 3 ml of PCA buffer instead of 10 ml. The viscosimetric assay was used for measuring cellulase activity.

In a second part of the experiment, Rhizobium trifolii strain BAL and the Rhizobium sp. "cowpea group" strains CB82 and CB1650 were grown in Bergersen's medium with 0.1% mannitol and 5 g/l of CMC. The inoculation, cultural conditions and enzyme extractions were performed as described above. Cellulase was determined by viscosimetry.

Effect of Nitrogen Source on Cellulase Production

The effect of N source on cellulase production was studied growing Rhizobium trifolii strains BAL and BART A in Bergersen's medium with myo-inositol (2% m/v) as C source and with each of two inorganic N sources (NH_4Cl 0.5% w/v and KNO_3 0.5% w/v) with or without sodium glutamate (0.11% w/v). The control treatment was glutamate alone as N source. The media were inoculated and incubated as described before. After 8 days of incubation EF enzyme extracts were prepared and cellulase activity measured by the viscosimetric assay.

Effect of Plant Root Extracts on Cellulase Production

To study the promotion of cellulase production by plant root substances and agar, Rhizobium trifolii strains BAL and BART A were grown in the presence of root extracts or agar (0.2% w/v, Difco agar, Difco Laboratories, Detroit, MI). For the preparation of the root extracts, seeds of Trifoliu repens var. Louisiana Nolin were surface sterilized and germinated in sterile storage dishes (80 mm height x 100 mm diameter). These dishes had stainless steel mesh as support and Fahraeus' minus N nutrient solution (Fahraeus, 1957). The seeds were germinated at room temperature under fluorescent lights. When the seedlings were 10 days old, the roots were cut with a razor blade and macerated in a mortar in PCA buffer over ice. The macerate was centrifuged at 15,300 x g for 15 min at 4°C. The supernatant (hereafter called root solubles) was sterilized by filtration using a 0.4 µm filter (Metricel, Gelman Scientifics, Ann Arbor, MI) and stored at

4°C. The pellet was resuspended in deionized water and frozen, thawed and centrifugated to wash soluble compounds. The procedure was repeated twice. After the last centrifugation, the pellet was resuspended in PCA buffer, autoclaved and stored at 4°C. The basal medium used was Bergersen's with myo-inositol (2% w/v) as C source (hereafter called INO medium; pH was adjusted to 7.0 with a solution of NaH_2PO_4 20% w/v) which received root solubles, root solids suspension or PCA buffer (5 ml in 100 ml of basal medium) or agar (0.2% w/v). The agar was added to the medium before autoclaving. All the others were filter sterilized and added to the medium after autoclaving. The media were inoculated and incubated as described previously. After 8 days of incubation, the cells were harvested by centrifugation and an EF enzyme extract prepared and tested for cellulase activity by both viscosimetry and release of reducing groups.

Effect of Mannitol on Cellulase Production

Mannitol is a carbohydrate commonly used in media for Rhizobium growth. Since readily available sugars usually repress the production of polysaccharide-degrading enzymes in microorganisms, the effect of the presence of mannitol in the culture medium was studied. Rhizobium trifolii strain BAL was grown in Bergersen's medium with different concentrations of mannitol (0.25, 0.50, 1.0, 1.5 and 2.0% m/v) with or without myo-inositol (1% w/v). The media were inoculated and incubated as described. After 8 days of incubation, an

EF enzyme extract was prepared and cellulase activity tested by the release of reducing groups.

Effect of the Culture Age on Cellulase Production

To study the production of cellulase with respect to time of culture incubation, Rhizobium trifolii strains BAL and BART A were grown in INO medium with or without agar (0.01% w/v). The medium was inoculated and incubated as described. At every sampling time (0, 1, 2, 4, 6 and 8 days), 2 flasks of each strain were randomly selected and an EF enzyme extract prepared from 70 ml of culture medium from each flask. Enzymatic activity was determined by viscosimetry.

Purification of Enzyme Activity

Cells from 6.35 liters of Rhizobium trifolii strain BAL culture (4 days) in INO medium were collected by centrifugation at 3,550 x g for 1 hour and resuspended in 120 ml of glycine-urea buffer solution (GU buffer solution composed of 0.2M K_2HPO_4 in 1% w/v glycine, 1M urea and 0.1M citric acid in 1% glycine and 1M urea mixed to give a pH of 7.5). Chloramphenicol and dithiothreitol (Sigma Chemical Co., St. Louis, MO) were added to a concentration of 100 µg/ml and 1mM, respectively. The cell suspension was sonicated twice for 1 min at 60W and centrifuged at 15,300 x g for 15 min at 4°C.

The protein of the supernatant was fractionated by $(NH_4)_2SO_4$ precipitation (Schwarz/Mann, Orangeburg, NY) in 20% saturation steps up to 80% saturation. Precipitation

was carried out adding solid salt in small quantities to the enzyme extract at 4°C under constant stirring.

The precipitates from each degree of saturation were redissolved and dialyzed in GU buffer solution with 0.05 µg/ml of $CaCl_2 \cdot 2H_2O$ for 5 hours in dialysis membranes with a molecular weight cutoff of 1,000 daltons (Spectra/Por 6, Spectrum Medical Industries, Los Angeles, CA) and at 4°C. The fractions which precipitated at 20%, 40% and 60% saturation, contained cellulase activity and were pooled. The pooled material was placed in a column (length 10.5 cm x diameter 5 cm) of DEAE cellulose (DE 52 preswollen and provided by the manufacturer in the free base form, Whatman, Clifton, NJ) stabilized with GU buffer solution. The column was eluted with a linear gradient of NaCl and pH at a flow rate of 0.96 ml/min. The gradient was from 0M NaCl pH 7.5 to 1M NaCl pH 4.8. Fractions of 4.6 ml were collected and assayed for cellulase activity by viscosimetry. The fractions which showed activity were pooled and the protein precipitated with $(NH_4)_2SO_4$ to 80% saturation at 4°C. The precipitate (hereafter called DEAE semipurified cellulase) was resolubilized in 10 ml of GU buffer solution and desalted by passing it through a column of BioGel P2 (Bio-Rad Laboratories, Richmond, CA; 12 x 2.5 cm) stabilized with GU buffer solution.

Effect of pH of Substrate on Cellulase Activity

Solutions of CMC with different pH values (pH 5, 6, 7 and 8) were prepared by addition of 5N NaOH. The viscosimetric

method was used to measure cellulase activity adding 0.5 ml of a 1:5 dilution of the DEAE semipurified cellulase to 5 ml of the CMC solution.

The experiment was also performed using Rhizobium trifolii strain BAL EF enzyme extract.

Effect of Calcium on Cellulase Activity

The effect of calcium was studied adding 0.1 ml of solution of $CaCl_2 \cdot 2H_2O$ at different concentrations (the final concentrations were 0, 0.46, 2.3, 4.5, 9.1 and 18.1 x 10^{-4}M) or of solution of ethylenediaminetetraacetic acid (EDTA) to 0.9 ml of EF enzyme extract. The cellulase activity was measured by the viscosimetric assay.

Effect of Magnesium on Cellulase Activity

The effect of magnesium on the Rhizobium cellulase activity was studied as previously described for calcium. Solutions with different concentrations of $MgSO_4 \cdot 7H_2O$ were used. The final concentrations of $MgSO_4$ were 0, 1.25, 2.5, 5, 10 and 20 µg/ml. The cellulase activity was measured by the viscosimetric assay.

Cellulase Production by Different Strains of Rhizobium

Several strains of Rhizobium trifolii (BAL, BART A, 0403, 0435, 0435-2, 521 and 521nod$^-$8) with different infectiveness on Trifolium repens were grown in INO medium. Inoculation and incubation were performed as described previously. After 8 days of incubation, the cells were harvested by centrifugation

and an EF enzyme extract prepared as described. Cellulase activity was measured by viscosimetry.

RESULTS AND DISCUSSION

Screening for Cultural Conditions for Cellulase Production

Table 2 shows the results of cellulase production by *Rhizobium* in different cultural conditions. The fast-growing *Rhizobium trifolii* strain BAL was more fastidious with respect to the C source for growth (Table 3). Bergersen's medium with myo-inositol was the best medium for cellulase production for this strain. The slow-growing "cowpea type" strain CIAT 79 grew well on most carbohydrates but showed cellulase activity only with xylose and gluconate, while *Rhizobium japonicum* strain J61 did not produce detectable cellulase activity in any of the media used. It is apparent that cellulase production is triggered by different inducers in different *Rhizobium* species.

Effect of the Concentration of Myo-inositol or Potassium Gluconate on Cellulase Production

Figure 1 shows cellulase levels in cells grown at different concentrations of myo-inositol. The cellulolytic activity increases dramatically at concentrations higher than 0.25% but there is not too much difference above 0.5%. Enzymatic activity was higher at a concentration of 2% than at 1%, albeit nonsignificantly, in spite of the fact that

TABLE 2: Cellulase production by Rhizobium growing in different media.

C Source	Basal medium					
	Bergersen's			RDM		
	Strains			Strains		
	BAL	CIAT 79	J61	BAL	CIAT 79	J61
Gluconate	- *	++	-	-	-	-
Xylose	-	+++	-	-	+++	-
Succinate	-	-	-	-	-	-
Arabinose	+	-	-	+	-	-
Myo-inositol	+++	-	-	-	-	-
Malate	-	-	-	-	-	-

* Enzymatic activity from the cup-plate assay:

- no enzymatic activity
+ traces of activity
++ light enzymatic activity
+++ clear enzymatic activity

TABLE 3: Growth of Rhizobium in two basal media with different C sources.

C Source	Basal medium					
	Bergersen's			RDM		
	Strains			Strains		
	BAL	CIAT 79	J61	BAL	CIAT 79	J61
Gluconate	3*	4	3	1	4	4
Xylose	0	5	1	0	4	0
Succinate	0	2	2	0	3	3
Arabinose	1	5	4	0	4	1
Myo-inositol	5	4	2	5	1	1
Malate	0	4	3	0	4	3

*Scale of microbial growth: 0 no growth to
5 prolific growth.

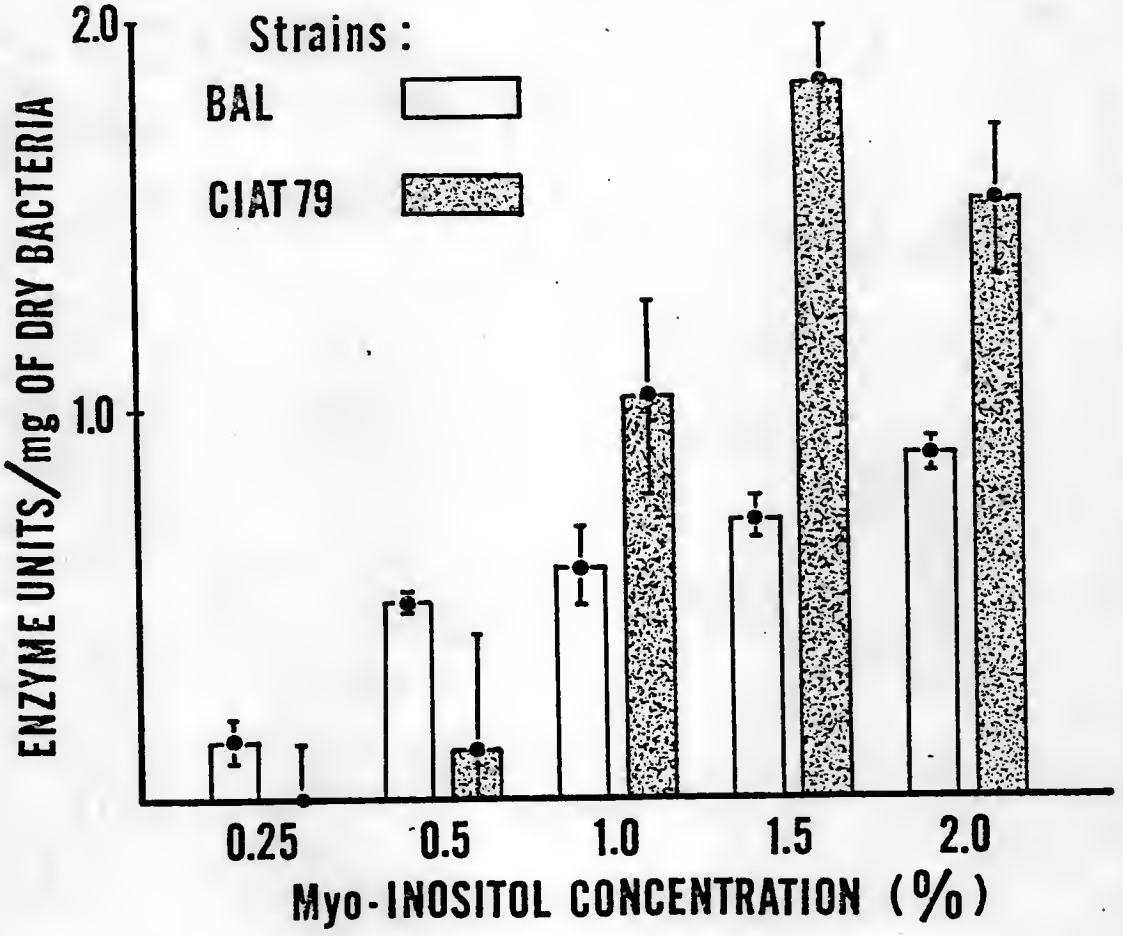


FIGURE 1: Effect of the concentration of myo-inositol in the culture medium on cellulase production by Rhizobium. Enzymatic activity was assayed by the increase of reducing groups in a CMC solution. For enzyme unit definition see Materials and Methods section. Vertical bars represent standard deviations.

Rhizobium trifolii grows faster and has a larger number of viable rhizobia at 1% myo-inositol than at 2% at harvest time. The reason may be that at 2% concentration of cyclitol there is a higher rate of death and lysis when the culture reaches the stationary phase, releasing the enzyme from the cells.

"Cowpea type" Rhizobium CIAT 79, which did not show detectable activity with myo-inositol by the cup-plate assay, had a higher activity than BAL when measured by the release of reducing groups (Figure 1). Enzyme extracts of this strain usually give small decreases in viscosity of CMC solution (Table 5). This might indicate the presence of an exoglucanase in the preparation. Unfortunately, CIAT 79 cultures have high levels of reducing sugars which are probably the cause of the high variation observed in the results of the Somogyi-Nelson assay.

Gluconate does not induce cellulase production in Rhizobium trifolii strain BAL (Figure 2) but it does with "cowpea type" rhizobia CIAT 79. Cellulase activity in CIAT 79 cultures with gluconate was more than twice that found in cultures with myo-inositol of the same strain as measured by the reducing groups assay. It seems, therefore, that cellulase is induced by different substances in fast- and slow-growing Rhizobium.

Cellulase Activity Induced by Different Carbohydrates

Rhizobium trifolii strain BAL produced cellulase equally with all the carbohydrates tested with the exception of xylan

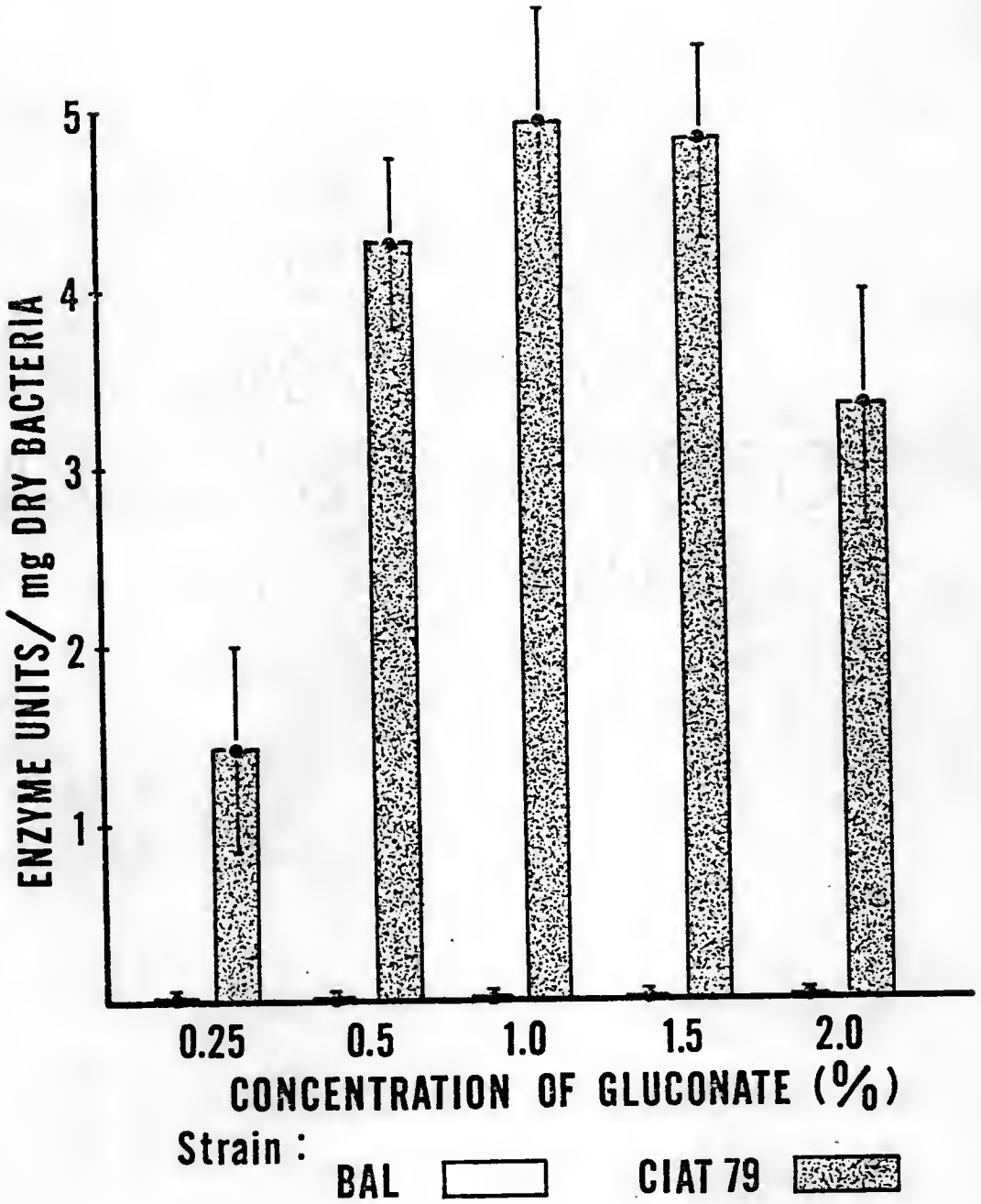


FIGURE 2: Effect of the concentration of potassium gluconate on cellulase production by Rhizobium. Enzymatic activity was determined by the increase in reducing groups in a CMC solution. For enzyme units definition see Materials and Methods section. Vertical bars represent standard deviations.

when NH_4Cl was the N source (Table 4). There was no difference between inducers in enzymatic activity or in growth.

Rhizobium sp. "cowpea type" CIAT 79 did not show significant cellulase production with most inducers tested using the viscosimetric assay (Table 5). Cellulase was detected in enzyme extracts of this strain only when growing with cellobiose and KNO_3 as N source. However, this is not a general feature of all "cowpea type" strains. Cultures of Rhizobium sp. strain CB82 showed considerable cellulase activity when growing with CMC and low levels of mannitol, while CB1650 is similar to CIAT 79 with respect to viscosity reduction (Figure 3). It seems that slow-growing Rhizobium strains differ in ability to produce cellulase. This is not surprising since "cowpea group" is very heterogeneous with differences in range of host plants, growth on artificial media and environmental conditions where they are found.

Effect of the Nitrogen Source on Cellulase Production

Cellulase production by Rhizobium trifolii strains BAL and BART A growing with different N sources is shown in Table 6. BAL showed the highest enzyme production per 100 mg of dry bacteria with NH_4Cl regardless of the presence of glutamate, but the yield of the enzyme per ml of enzyme extract was 20 times lower than with glutamate alone. This probably was an effect of the poor growth displayed by BAL with mineral salts (data not shown). BART A, by contrast, grew more slowly with mineral N than with glutamate alone but produced the

TABLE 4: *Rhizobium trifolii* strain BAL production of cellulase with different carbohydrates and N sources in the culture medium.

Carbohydrate	N source		
	NH_4Cl	KNO_3	Na glutamate
CMC	82*	86	73
Xylan	0	76	63
Locust bean gum	73	78	62
Gum arabic	70	69	69
Cellobiose	78	79	47

NOTE: The control treatment consisted of the mixture of 1 ml PCA buffer and 5 ml of CMC solution, and incubated as described. Control showed a 2% decrease in viscosity after 24 hours.

*Percentage of viscosity decrease after 24 hours of incubation. Average of two replications.

TABLE 5: Rhizobium sp. "cowpea group" strain CIAT 79 production of cellulase with different carbohydrates and N sources in the culture medium.

Carbohydrate	N source		
	NH_4Cl	KNO_3	Na glutamate
CMC	11*	4	4
Xylan	3	7	6
Locust bean gum	9	3	4
Gum arabic	5	4	5
Cellobiose	2	20	8

NOTE: For the control treatment 1 ml of PCA buffer was mixed with 5 ml of CMC solution and incubated as described. Control showed a 3% decrease in viscosity in 24 hours.

*Percentage of viscosity decrease after 24 hours of incubation. Average of two replications.

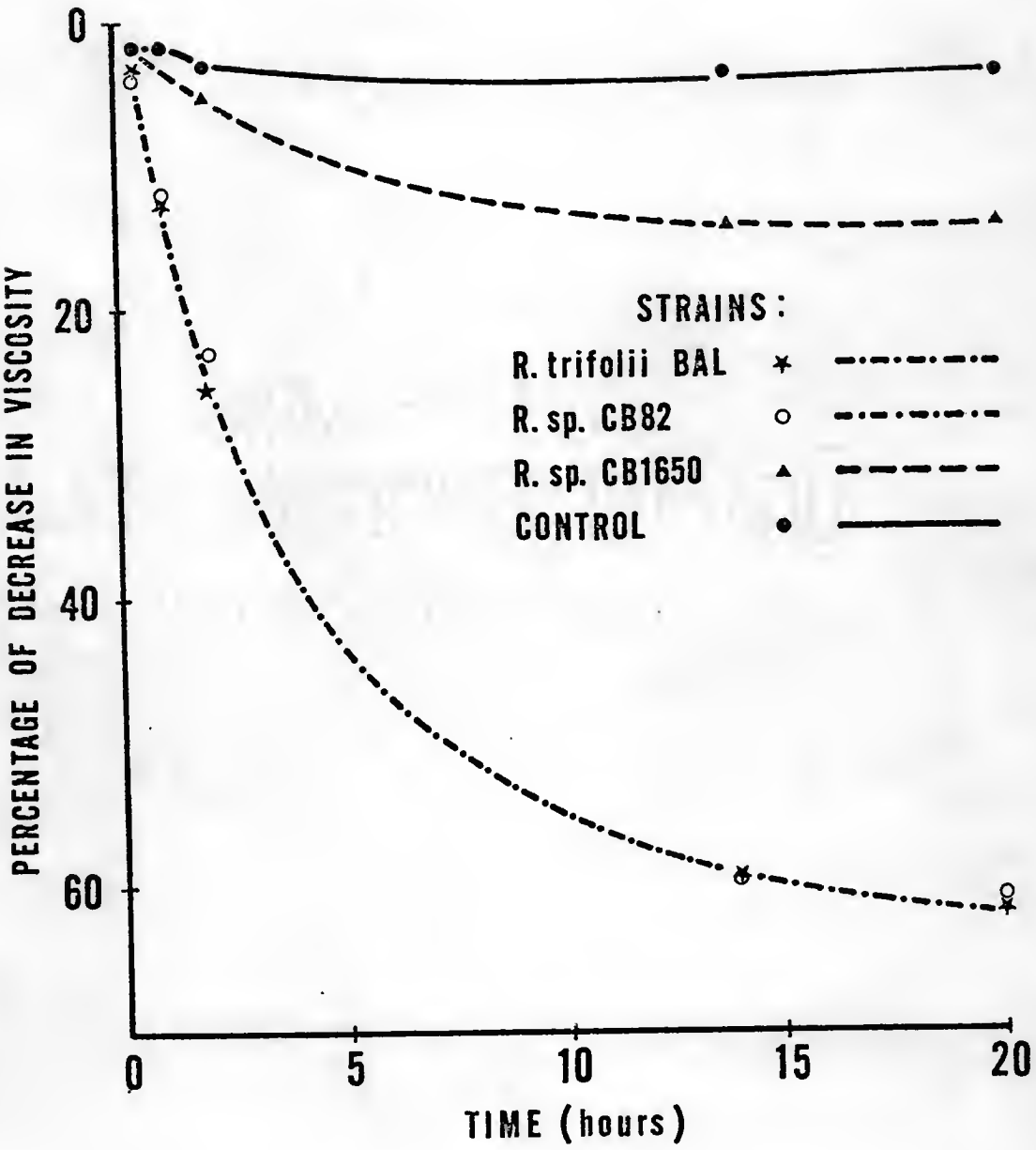


FIGURE 3: Cellulase activity of enzyme extracts of different Rhizobium strains. The strains were grown for 8 days, in Bergersen's basal medium with 5 g/l of CMC and 0.1% w/v myo-inositol. Each point is the average of 2 replications. Boiled BAL enzyme extract was used as control treatment.

TABLE 6: Effect of the N source of the culture medium on cellulase production by Rhizobium trifolii strains BAL and BART A.

N Source	Strain			
	BAL		BART A	
	Enzyme Units			
	Per volume*	Per mass**	Per volume	Per mass
NH_4Cl with glutamate	0.06	47	2.00	190
NH_4Cl without glutamate	0.07	160	0.00	0
KNO_3 with glutamate	0.00	0	1.84	131
KNO_3 without glutamate	0.00	0	0.00	0
Glutamate only	1.22	28	1.54	118

NOTE: All cultures were processed to obtain the same volume of enzyme extract. Cellulase was assayed by the viscosimetric method. Each value is the average of two replications. For definition of enzyme unit see the Materials and Methods section.

*Enzymatic activity expressed as EU/ml of enzyme extract.

**Enzymatic activity expressed as EU/100 mg of dry bacteria.

same amount of enzyme when growing with glutamate with or without mineral N salts. In this strain, then, enzyme production was directly proportional to the growth.

Effect of Root Extracts on Cellulase Production

The root solids solution showed a protein content of 32 µg/ml (by the Lowry assay) and a total carbohydrate content of 71 µg/ml (by the phenolsulfuric acid method).

Root extracts seem to stimulate the production of cellulase by strain BAL (Table 7). Root solids showed a striking stimulation of enzyme production as measured by the release of reducing groups. However, the same stimulation was not observed in the results of the viscosimetric assay (Table 8). In BAL cultures with root solids, cells tended to adhere to the root pieces and did not grow as well as in other treatments. No effect was shown by the addition of root extracts on BART A growth or cellulase production as measured by viscosimetry, but the release of reducing groups was strongly stimulated by root solids and, to a lesser extent, by agar. The difference in results obtained with these two assays may indicate that a different set of enzymes is produced by different treatments. Enzyme extracts from cultures with root solids and agar showed delayed or no pigment production (see Appendix II for discussion on pigment production) and also low content of reducing sugars.

TABLE 7: Effect of root extracts on cellulase production by Rhizobium trifolii strains BAL and BART A.

Culture amendment	Strain			
	BAL		BART A	
	Enzyme units			
	Per volume*	Per mass**	Per volume	Per mass
Root solubles	1.9	29.6	1.8	85.9
Root solids	2.9	161.8	12.0	513.2
Agar	0.7	12.2	4.7	227.1
No amendment	1.0	16.0	2.3	111.3

NOTE: Enzymatic activity was measured by the release of reducing groups assay. Each value is the average of 4 replications. For definition of enzyme unit see the Materials and Methods section.

*Enzymatic activity expressed as EU/ml of enzyme extract.

**Enzymatic activity expressed as EU/100 mg of dry bacteria.

TABLE 8: Effect of root extracts on cellulase production by Rhizobium trifolii strains BAL and BART A.

Culture amendment	Strain			
	BAL		BART A	
	Enzyme units			
	Per volume*	Per mass**	Per volume	Per mass
Root solubles	1.1	16.4	1.7	79.7
Root solids	0.3	15.4	1.2	52.9
Agar	0.4	6.6	1.2	58.1
No amendment	0.4	6.0	1.4	68.6

NOTE: Enzymatic activity was assayed by the viscosimetric method. Each value is the average of 2 replications. For definition of enzyme unit see the Materials and Methods section.

*Enzymatic activity expressed as EU/ml of enzyme extract.

**Enzymatic activity expressed as EU/100 mg of dry bacteria.

Effect of Mannitol on Cellulase Production

Mannitol in the culture medium inhibits the production of cellulase (Table 9). In the presence of myo-inositol there was more cellulase produced at a concentration of mannitol of 0.25% than at higher concentrations. When mannitol is the only C source, cellulase production drops steadily as mannitol concentration increases. Above 1.5% mannitol there is only a very small enzymatic activity. The high enzyme concentration when bacteria were grown with mannitol as the only C source is a result of the correction for bacterial growth; actually the enzyme concentration per ml of enzyme extract in these treatments is low. The results indicate that cellulase production is controlled. However, no reason seems apparent for myo-inositol to be an inducer or mannitol to be a repressor; both substances are readily metabolized by the microbe. It may be that Rhizobium once possessed the capability not only of synthesizing but of degrading and utilizing cellulose for growth, and they partially lost the capability for degradation. Myo-inositol, being commonly present in plant tissues (Loewus and Loewus, 1980), could act as an inducer of production of polysaccharide-degrading enzymes. But, it would be uncommon that a microorganism, in the highly competitive soil environment, would retain genes which do not contribute to its ability to survive.

Effect of Age of Culture on Cellulase Production

Figures 4 and 5 show cellulase production with respect to age of culture. The enzymatic activity is expressed in

TABLE 9: Effect of the concentration of mannitol on the production of cellulase by Rhizobium trifolii strain BAL.

Mannitol concentration -%-	Myo-inositol concentration -%-	Enzyme units (EU) Per volume*	Standard deviation	Per mass**	Standard deviation
0.25	1	2.9	0.5	36.2	6.2
0.50	1	1.2	0.8	15.0	10.0
1.00	1	1.2	0.3	15.0	3.7
1.50	1	1.1	0.6	14.1	7.7
2.00	1	0.8	0.3	10.2	3.8
0.25	0	1.0	0.3	40.0	12.0
0.50	0	0.8	0.3	25.8	9.7
1.00	0	0.6	0.3	16.0	8.0
1.50	0	0.0	0.2	0.0	6.8
2.00	0	0.3	0.3	2.7	7.7

NOTE: Enzymatic activity was measured by the release of reducing groups. Each value is the average of 4 replications. For definition of enzyme unit see the Materials and Methods section.

*Enzymatic activity expressed as EU/ml of enzyme extract.

**Enzymatic activity expressed as EU/100 mg of dry bacteria.

FIGURE 4: Cellulase activity per dry weight of bacteria of cultures of Rhizobium trifolii strains BAL and BART A at different times of culture incubation. Enzymatic activity was measured by the release of reducing groups assay. For enzyme unit definition see the Materials and Methods section.

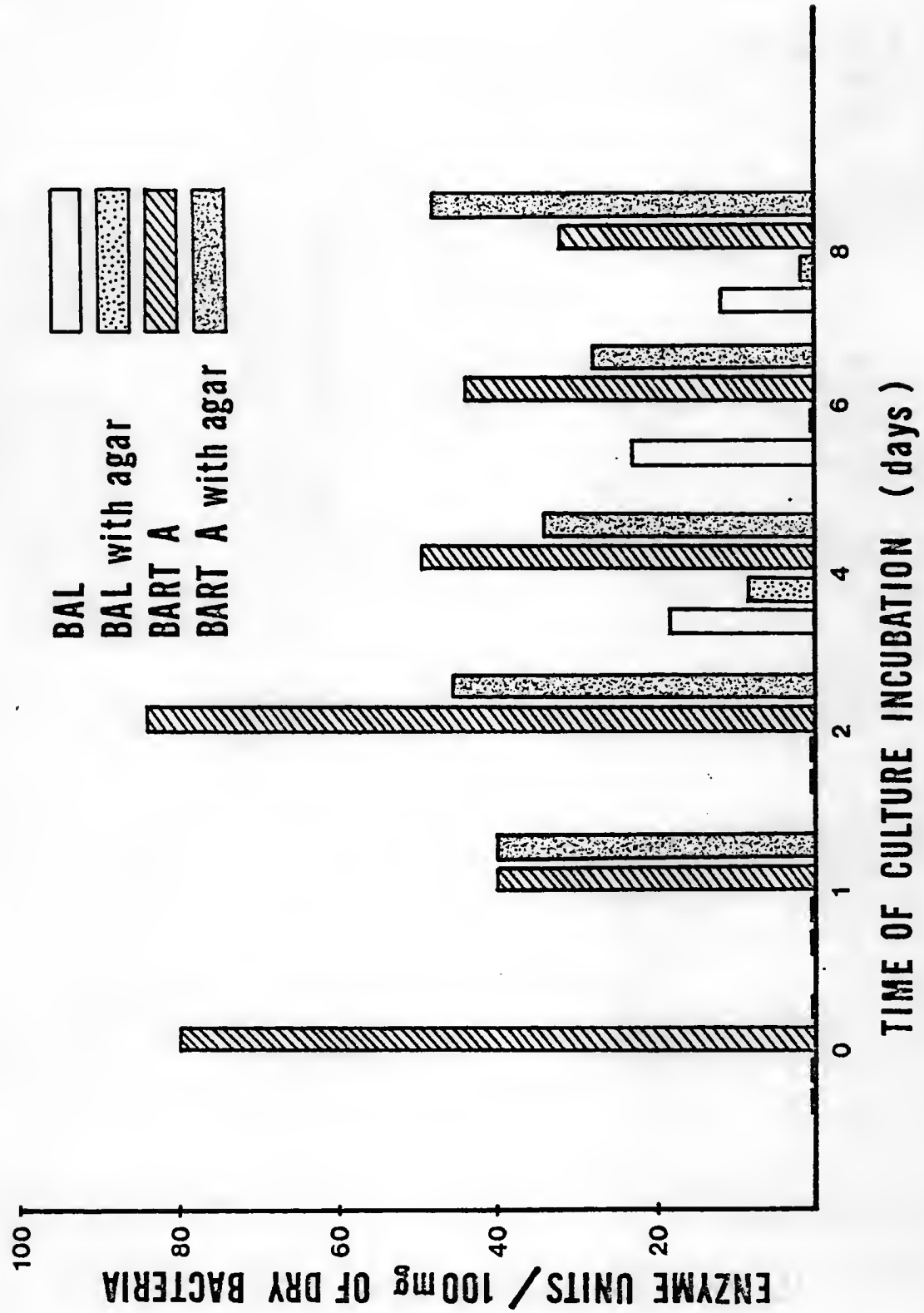

BAL
BAL with agar
BART A
BART A with agar
ENZYME UNITS / 100 mg OF DRY BACTERIA
100
80
60
40
20
0
1
2
4
6
8
TIME OF CULTURE INCUBATION (days)

FIGURE 5: Cellulase activity per volume of enzyme extract of cultures of *Rhizobium trifolii* strains BAL and BART A at different times of culture incubation. Same data as Figure 4 but on a volume basis.

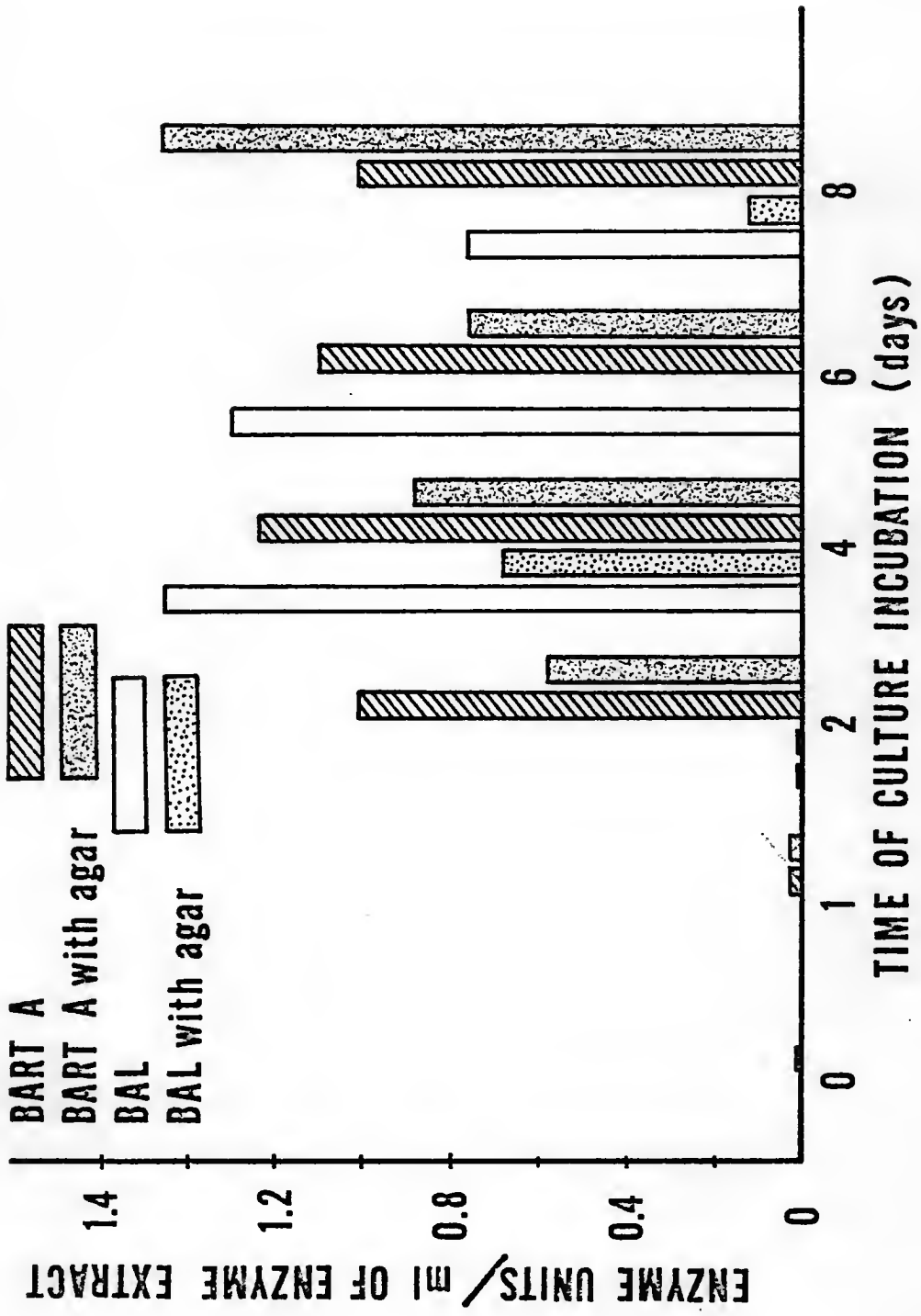

BART A
BART A with agar
BAL
BAL with agar
ENZYME UNITS/ml OF ENZYME EXTRACT
0
0.4
0.8
1.2
1.4
0
1
2
4
6
8
TIME OF CULTURE INCUBATION (days)

Figure 4 on the basis of dry weight of bacteria while Figure 5 shows the same results expressed per ml of enzyme extract. The two strains differ with respect to cellulase production. BART A produces cellulase earlier than BAL. BART A cultures showed traces of activity after 1 day of incubation while BAL cultures did so after 2 days of incubation.

The activity expressed on a basis of dry weight of bacteria may be misleading, especially with young cultures. A trace of activity in cultures with very small dry weight of bacteria per ml will give a high activity per mg of dry bacteria.

The peak of production of enzyme as expressed in Figure 4 occurs after 2 days of incubation for BART A and after 6 days for BAL. At these ages the cultures are in early stationary phase (see Appendix II). The highest rate of extractable enzyme occurs after 4 days of incubation with the exception of BART A growing with agar (Figure 5). Agar seems to depress the production of cellulase, but this could be due to the higher viscosity of the cultures with agar which could make sonication less efficient in releasing the enzyme, or the enzyme may bind to the agar and not be extracted. All Rhizobium trifolii strains tested release a yellow pigment when growing in INO medium after reaching early stationary phase (unpublished results). Cultures of BART A with agar start producing the pigment one day before cultures without agar. In contrast, pigment production is delayed in BAL cultures with agar. BAL cultures in INO medium show clumping. The

clumps are very numerous, of uniform size and shape. They are not affected by divalent cations, EDTA, detergents, trypsin, pectinase or cellulase. Only sonication disrupts them. BAL cultures with agar do not produce this clumping.

Cellulase production increases with time of incubation, reaches a peak and declines, with the exception of BART A in medium with agar, which showed a continuous upward trend.

Enzyme Purification

Most of the cellulase produced by Rhizobium is loosely bound to the cell surface and/or the capsular material. There is also a small detectable activity in the culture supernatant (ranging from 0.05 to 0.2 enzyme units/ml). This low enzyme concentration in such a large volume accounts for a good proportion of the total enzyme produced. But, it is very difficult to separate the enzyme from the extracellular polysaccharide. Of the total amount of enzyme (as measured by the viscosimetric assay) that remains with the cells after centrifugation, 40% is released into the buffer solution after sonication, 20% can be released after repetition of the extraction procedure and 40% remains attached to the cells.

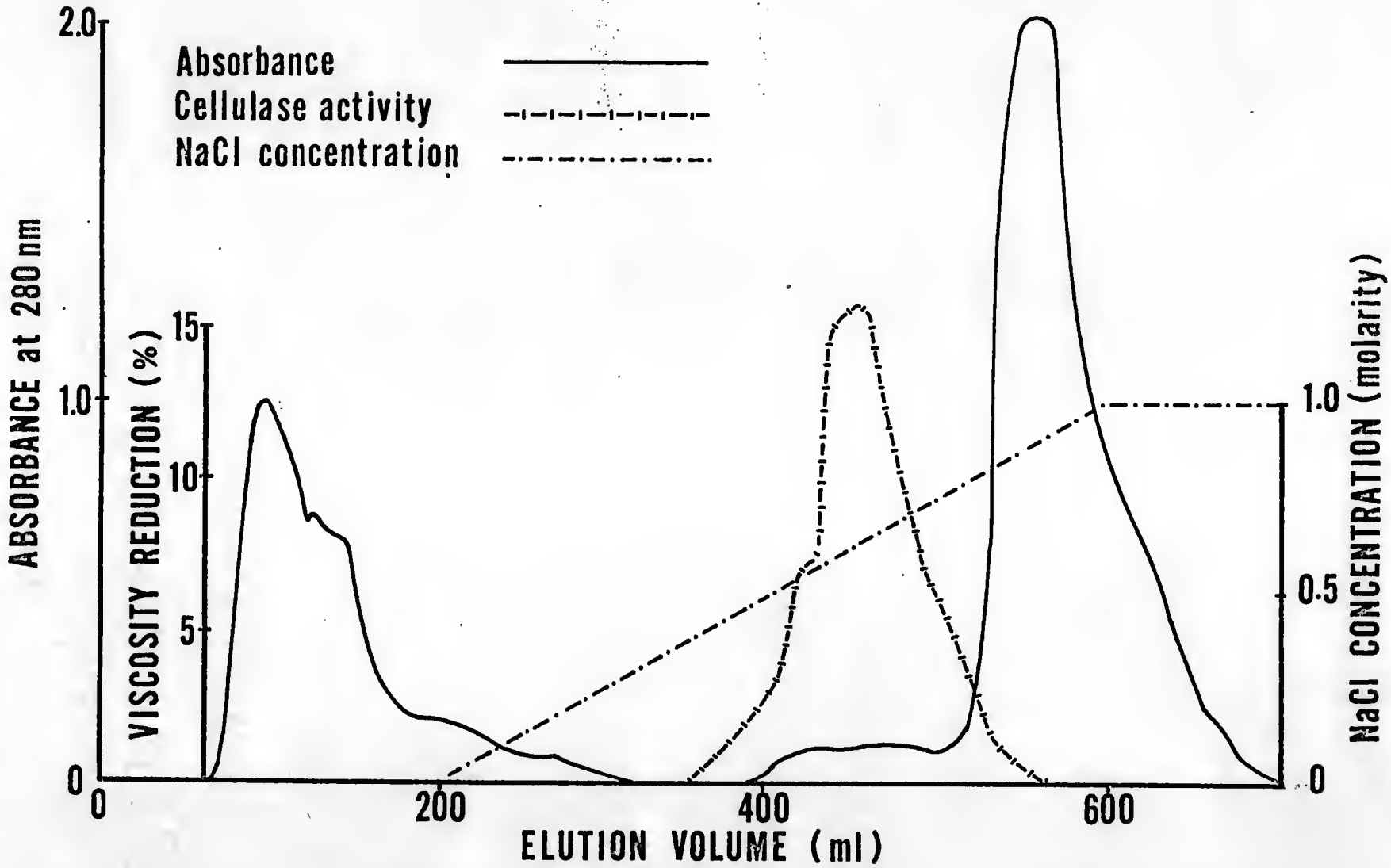
The type of extracting solution used influences the amount of enzyme released. The highest recovery was obtained with deionized water. PCA and GU buffers extract around 60% of the amount extracted with water. Tris HCl buffer (pH 8.0) releases only 20% of this amount. Enzyme extracts made up with water maintain the enzymatic activity for several weeks

when stored at 4°C or for 2 or 3 months when sterilized by filtration (polycarbonate filters, 0.4 μm, Gelman Industries, Ann Arbor, MI) and stored at 4°C.

When ammonium sulfate precipitation is performed slowly (allowing the solution to stand for at least 5 hours between steps), the fractions with 20%, 40% and 60% saturation show cellulase activity after dialysis. When a quick precipitation is performed, the fraction with 80% saturation also shows activity. GU buffer solution was essential for maintaining enzymatic activity. Without glycine and urea the precipitate did not dissolve completely and usually part of it reprecipitated after a few hours. A small amount of $CaCl_2 \cdot 2H_2O$ (0.05-0.1 g/ml) helped to solubilize the protein. Dialysis time should be short (4-5 hours; 10 hours maximum). Extensive dialysis resulted in complete loss of cellulase activity. This may be due to binding of the enzyme to the cellulosic dialysis membranes or denaturation. Several procedures were tested to recover enzyme from the surface of the dialysis membranes without success. The affinity of the enzyme for cellulosic material seems to increase as the ionic strength of the solution decreases.

Figure 6 shows the elution pattern of proteins from a DEAE cellulose column. Cellulase activity appeared in fractions eluting immediately before the main protein peak. Elution should be performed with a gradient of salt and pH. A salt gradient alone does not release the protein from the column.

FIGURE 6: Elution pattern of Rhizobium trifolii strain BAL cellulase after DEAE cellulose column chromatography. Cellulase activity is expressed as percentage of viscosity reduction of a CMC solution.


Absorbance
Cellulase activity
NaCl concentration
ABSORBANCE at 280 nm
VISCOSITY REDUCTION (%)
NaCl CONCENTRATION (molarity)
ELUTION VOLUME (ml)
2.0
1.0
0
15
10
5
1.0
0.5
0
0
200
400
600

Desalting after ammonium sulfate precipitation using a BioGel P2 column gave two peaks (Figure 7). The first peak showed low cellulase activity. This enzymatic activity disappeared in less than 24 hours, indicating that the enzyme is denatured under these conditions. Mixing the proteins from the two peaks did not restore cellulase activity.

Effect of pH of the Substrate on Cellulase Activity

Rhizobium cellulase increases activity with an increase in pH (Figure 8). The highest activity was found at pH 8.0, which was the highest pH tested. However, the increase was mainly on the initial rate of activity and was relatively small.

Assays with DEAE semipurified enzyme did not show any effect of pH on enzymatic activity. This was probably due to the presence of urease in the preparation. Urease activity, although small, increased the pH during incubation.

Effect of Calcium on Cellulase Activity

Calcium did not show effect on the activity of Rhizobium cellulase. Although the enzymatic activity was higher when calcium was present, the differences were not significant (Figure 9). There was no difference either when EDTA was added to the CMC solution or when the $CaCl_2 \cdot 2H_2O$ concentration was increased to 4.5×10^{-2} M.

FIGURE 7: Elution pattern of Rhizobium trifolii strain BAL cellulase after desalting through a column of BioGel P2. Cellulase activity expressed as percentage of viscosity reduction.

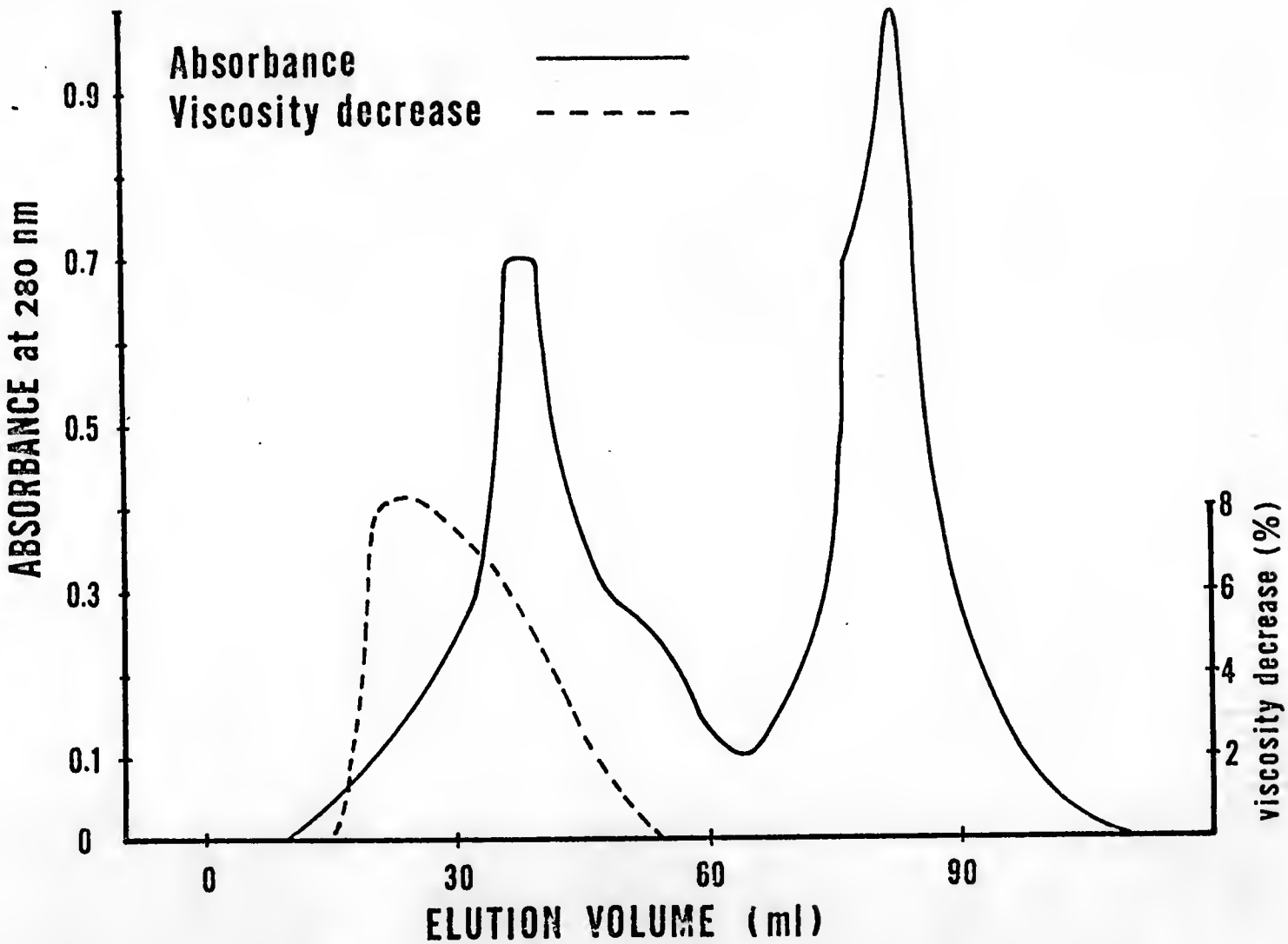
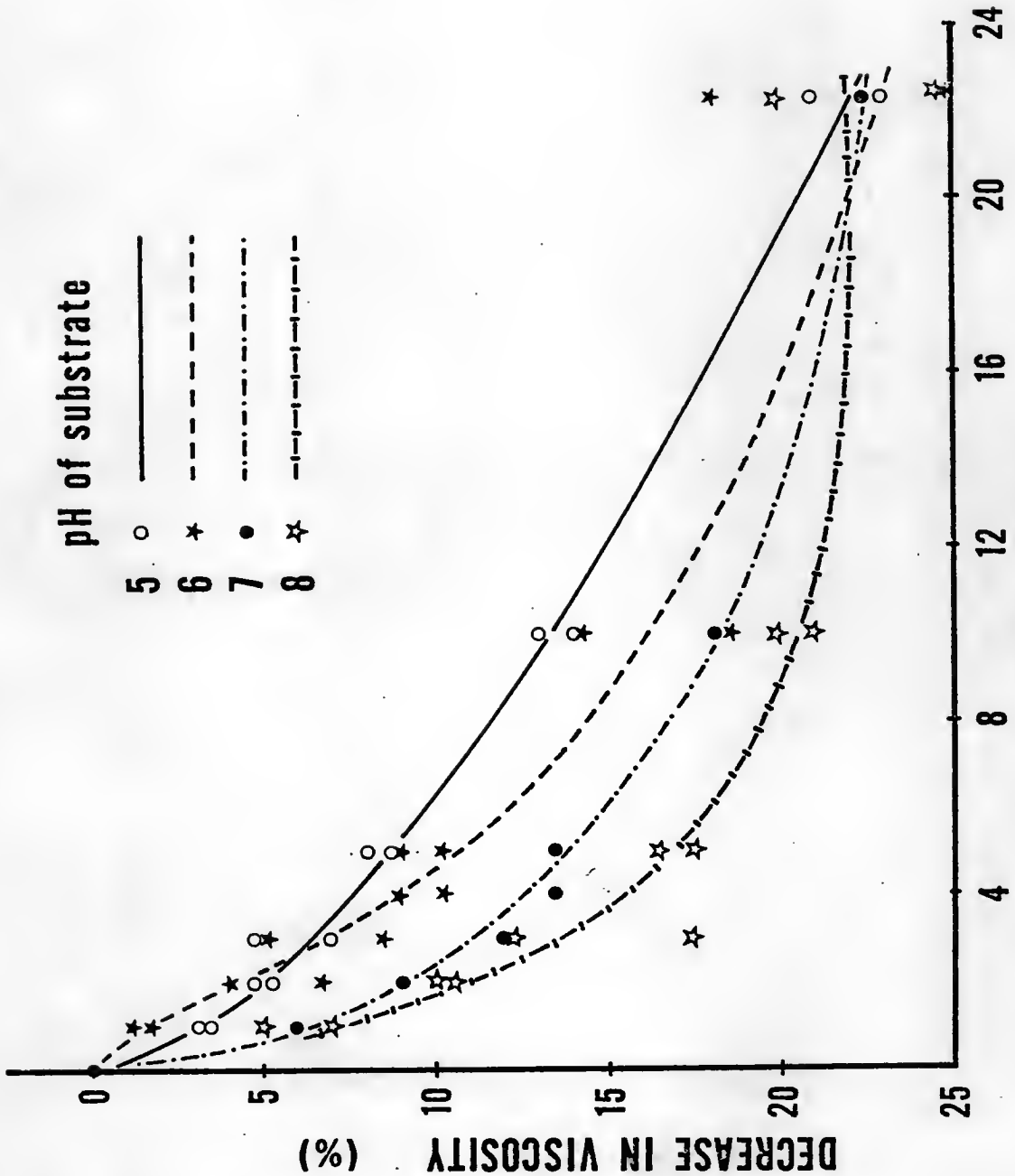

Absorbance
Viscosity decrease
ABSORBANCE at 280 nm
0.9
0.7
0.5
0.3
0.1
0
0
30
60
90
ELUTION VOLUME (ml)
8
6
4
2
viscosity decrease (%)

FIGURE 8: Effect of the pH of the substrate on enzymatic activity of *Rhizobium trifolii* enzyme extract. Cellulase activity was assayed by the viscosimetric method.


pH of substrate
5
6
7
8
DECREASE IN VISCOSITY (%)
0
5
10
15
20
25
4
8
12
16
20
24
TIME (hours)

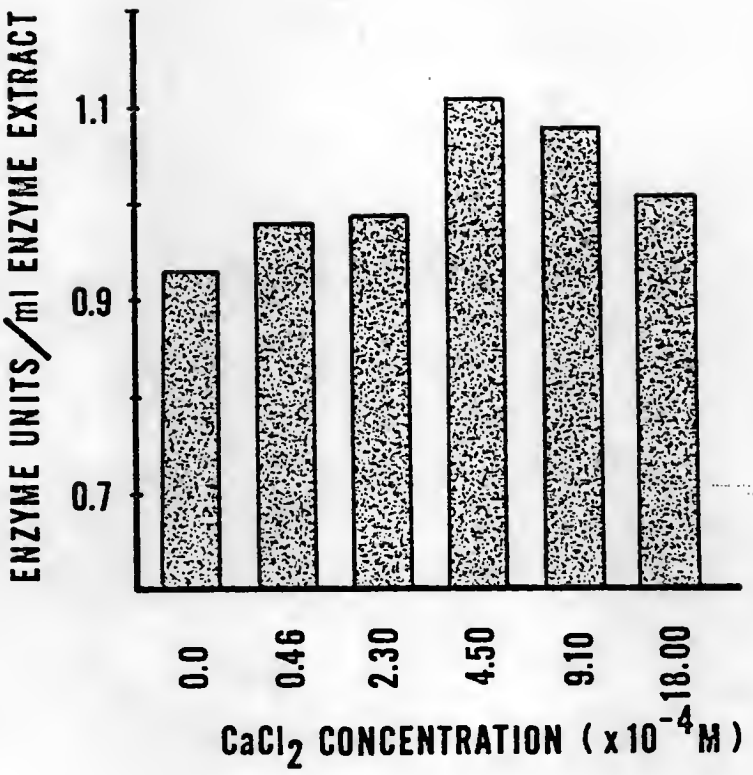


FIGURE 9: Effect of $CaCl_2$ on cellulase activity of Rhizobium trifolii enzyme extract. Enzymatic activity was assayed by the viscosimetric method. Each value is the average of 2 replications.

Effect of Magnesium on Cellulase Activity

Figure 10 shows the cellulase activity of Rhizobium trifolii strain BAL enzyme extract. There is not a clear trend in the changes of cellulase activities with different concentrations of magnesium. Magnesium did not affect enzymatic activity either alone or mixed with calcium (data not shown).

Enzyme Characterization

Figure 11 shows the plot of decrease in viscosity of a solution of CMC and the release of reducing groups caused by a Rhizobium trifolii strain BAL enzyme extract. There was a slow release of reducing groups. Eight hours of incubation were required to liberate the equivalent of 0.11 mmoles of glucose during which the viscosity of the solution decreased by 29%. This indicates that the enzyme is an 1,4-β-D-glucan 4-glucanohydrolase (endo-1,4-β-D-glucanase). CMC is degraded to oligosaccharides rather than to glucose or cellobiose. Glucose production was not detected by the glucose oxidase assay upon incubation with enzyme extract. Rhizobium trifolii is able to grow on cellobiose as sole C source and, therefore, must be able to produce cellobiase (β-glucosidase); however, it is unable to grow on CMC, indicating that no cellobiose was formed.

The relatively low activities observed and the difficulty of increasing it by purification may indicate that CMC is not the natural substrate for the enzyme. Enzymatic activities

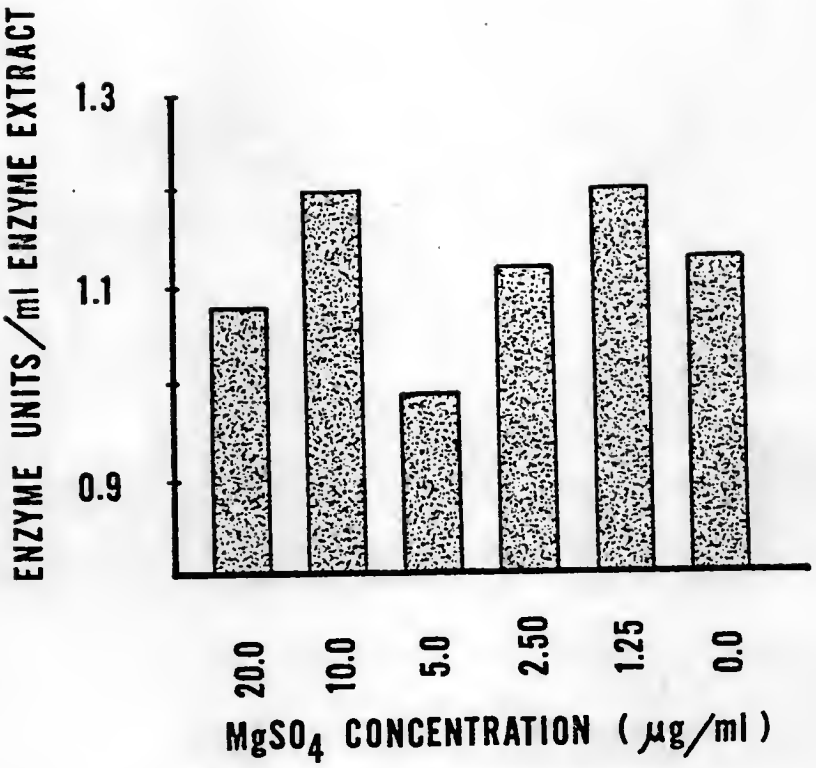
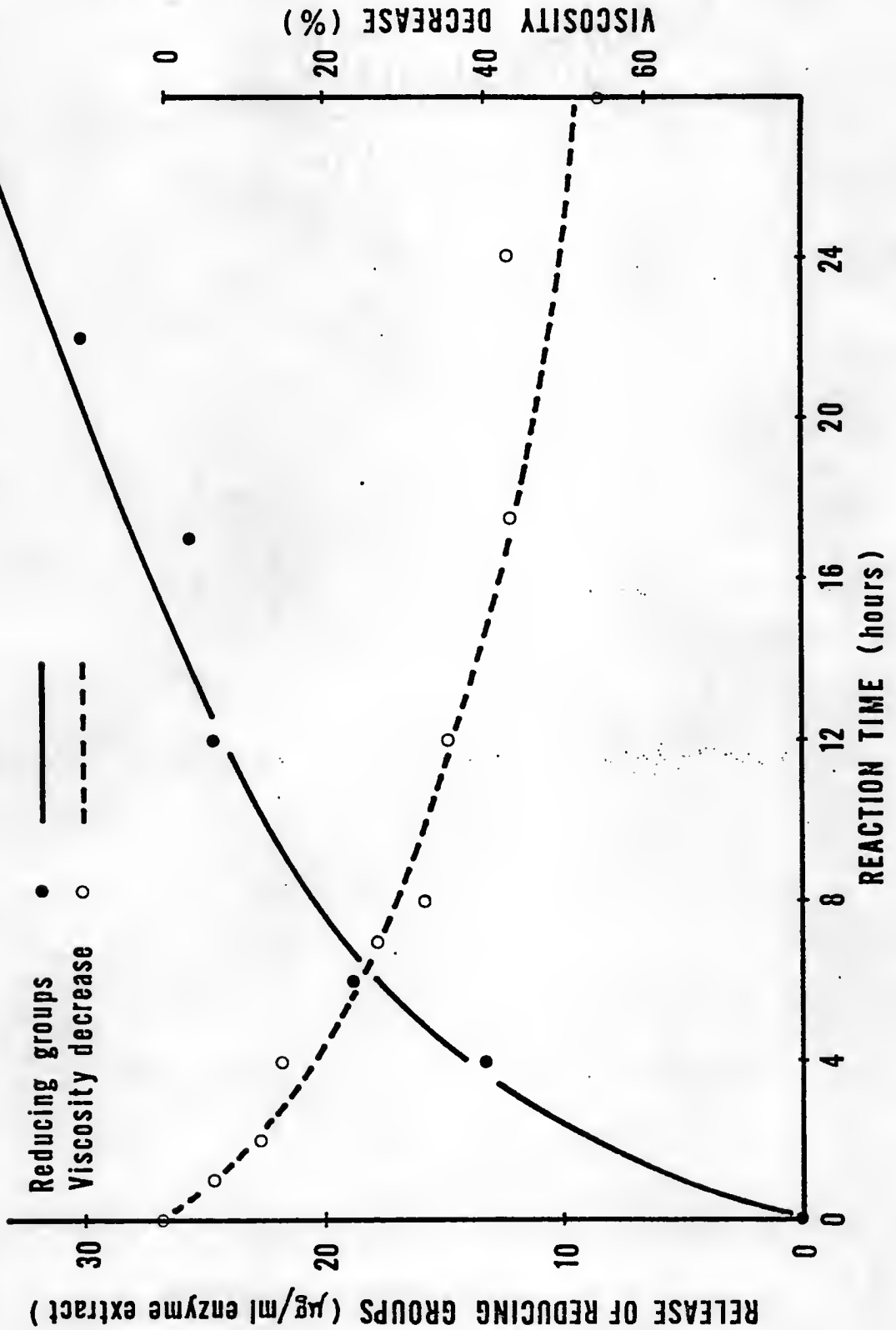


FIGURE 10: Effect of $MgSO_4$ on cellulase activity of Rhizobium trifolii enzyme extract. Enzymatic activity was assayed by the viscosimetric method. Each value is the average of 2 replications.

FIGURE 11: Comparison of plots of reduction of viscosity and release of reducing groups by Rhizobium trifolii enzyme extract. Each viscosity reduction value is the average of 2 replications. Each value of release of reducing groups is the average of 4 replications.


RELEASE OF REDUCING GROUPS (μg/ml enzyme extract)
VISCOSITY DECREASE (%)
REACTION TIME (hours)
Reducing groups
Viscosity decrease
0
10
20
30
0
20
40
60
0
4
8
12
16
20
24

on xylan, locust bean gum and gum arabic have been detected but at an even lower level than cellulase.

Rhizobium cellulase is resistant to heat. Overnight incubation at 30°C did not affect enzymatic activity; furthermore, increases in enzymatic activity have been observed after this treatment. Heating at 60°C for 30 min destroyed 70% of the activity while 15 min of boiling destroyed 95% of the cellulase.

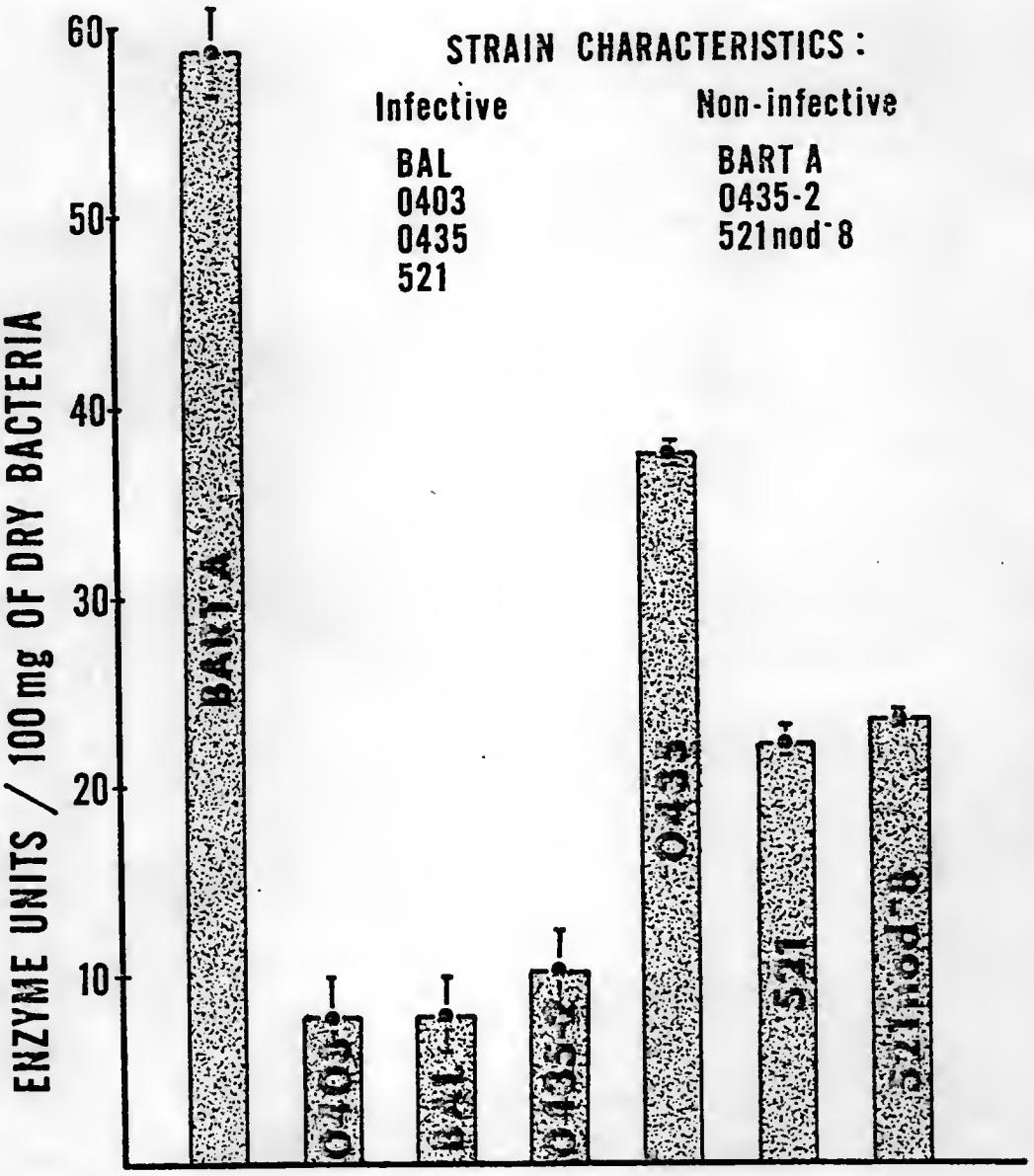
Production of Cellulase by Different Rhizobium trifolli Strains

Figure 12 shows the level of cellulase production by strains of Rhizobium trifolii with different infectiveness. There were differences between pairs of infective and non-infective strains. BART A, a noninfective derivative, produced 6 times more cellulase than the infective parental strain 0403 or BAL. All of these three strains possess three plasmids (Taylor, 1981), but BART A has a deletion in the sym plasmid. As a contrast, 0435, which is infective, produced almost 4 times the amount of cellulase produced by 0435-2, its noninfective derivative.

The strain 521nod$^-$8 is a derivative of 521, cured of the sym plasmid (190 x 10^6 daltons) (Zurkowski and Lorkiewicz, 1979). The two strains did not show any difference in cellulase production in the cultural conditions used.

The results obtained indicate that the gene(s) coding for the Rhizobium endoglucanase is not in the sym plasmid. Hooykaas et al. (1981) showed that the transfer of the sym

FIGURE 12: Production of cellulase by different strains of *Rhizobium trifolii*. Enzymatic activity was measured by the viscosimetric assay. Each value is the average of 4 replications.


STRAIN CHARACTERISTICS :
Infective
BAL
0403
0435
521
Non-infective
BART A
0435-2
521 nod⁻8
ENZYME UNITS / 100 mg OF DRY BACTERIA
60
50
40
30
20
10
BART A
0403
BAL
0435-2
0435
521
521 nod⁻8

plasmid of Rhizobium trifolii strain LPR 5001 (180 x 10^6 daltons) to a Ti plasmid cured derivative of Agrobacterium tumefaciens makes the transcojugants to induce nodule formation in clover roots. The nodules were white, nonefficient and appeared after a long period of incubation.

The results do not rule out the possibility of a role of these endoglucanases in other stages of nodule development such as bacteroid differentiation or the release of the bacteria from the infection thread. They could also be important in the infection process of tropical legume roots which are nodulated through a different pathway of infection (Chandler, 1978).

CONCLUSIONS

Cellulase production by Rhizobium strains is inducible. Cellulase was produced by Rhizobium trifolii strains when growing in Bergersen's medium with myo-inositol or cellobiose as C sources; or when one of a variety of polysaccharides was present in the medium. Enzyme production was repressed by mannitol at concentrations higher than 0.25% w/v. Slow-growing "cowpea type" Rhizobium strains produced cellulase (in all conditions tested), but the highest production was found when potassium glutamate was the C source.

Rhizobium trifolii strains produce an endo-β-glucanase which degrades CMC to oligosaccharides but not to cellobiose. There are indications that "cowpea type" rhizobia also produce an exo-β-glucanase.

Cellulase activity in raw enzyme extracts is stable, but decreases when carbohydrates and ions are removed. The presence of glycine, urea, calcium and magnesium helps to maintain the activity during the purification process, but fails to do so in the latter stages of purification.

Root substances stimulate the production of cellulase by Rhizobium trifolii strains. Strains growing in the presence of root solids showed higher cellulase activity, as

assayed by the release of reducing groups, than do cultures growing with root solubles or without amendment. However, cultures growing with root solubles had the highest activity when assayed by the viscosimetric method. This indicates that a different set of enzymes is induced by each root fraction.

Cellulase activity of enzyme extracts increases as pH of the substrate increases up to pH 8, which was the highest level tested. No effect on activity by calcium or magnesium was observed.

There was no correlation between ability to produce endo-β-glucanase by Rhizobium trifolii strains and infectivity of white clover. One of the strains lacking the nodulation plasmid proved capable of producing cellulase at the same rate as its infective parental strain.

APPENDIX I
VISCOSIMETRIC ASSAY FOR CELLULASE ACTIVITY

Amongst the various assays used for cellulase activity determination, the viscosimetric method has the advantages of being rapid and extremely sensitive, especially for endoglucanases. Its main disadvantage is that enzymatic activity is measured not in absolute terms but in relation to a physical change, a decrease in viscosity. The viscosity of a polymer solution is a function of its average molecular weight. Viscosimetric assays can indeed, after certain factors are controlled, measure enzymatic activity in absolute terms (Almin and Eriksson, 1967). Most workers, however, express the results in relative terms, either as percentage of decrease of viscosity per unit of time, time required for attaining a 50% decrease in viscosity, or the slopes of the straight lines resulting from plotting reciprocal specific viscosities ($1/\eta_s$) against reaction time (Rexova-Benkova and Markovic, 1976). The fact that Rhizobium cellulase activities are very low necessitated testing the reliability of the viscosimetric assay.

Figure 13 shows the relation between enzyme amount and the slope of the line of the plot of reciprocal specific viscosities against reaction time (hereafter called slope $1/\eta_s$).

The different amounts of enzyme were obtained by dissolving a Rhizobium trifolii strain BAL enzyme extract with water. An enzyme unit was defined as the amount of cellulase that reduces the viscosity of a CMC solution (0.2% w/v) by 50% in 10 hours or has a slope $1/\eta_s$ of 1.883×10^{-2} hours^{-1}, which is approximately equivalent to the liberation of reducing groups equal to 25 µg of glucose in 24 hours.

The graph (Figure 13) indicates that the relationship is linear above 0.2 EU. A regression calculated with the data obtained with the enzyme amounts ranging from 0.3 to 1.5 EU produced the following equation:

$$EU = -0.93 + 1.51 \log(\text{slope } 1/\eta_s \times 1{,}000)$$

Correlation = 0.9996

Figure 14 shows the plot of enzyme amounts against time for achieving 50% of viscosity reduction. The relationship is that of a straight line above 0.3 EU. For enzyme quantities smaller than 0.6 EU, the time to achieve 50% viscosity reduction is more than 20 hours. In practice, these times should be calculated by extrapolation from a plot of $1/\eta_s$ vs time of reaction because incubations longer than 24 hours are not reliable. Therefore, the best way to calculate low cellulase activities is through calculation of the slopes $1/\eta_s$.

FIGURE 13: Plot of the relationship between cellulase amount and the slope of the reciprocal of the viscosity vs time of reaction. Each point is the average of 2 replications.

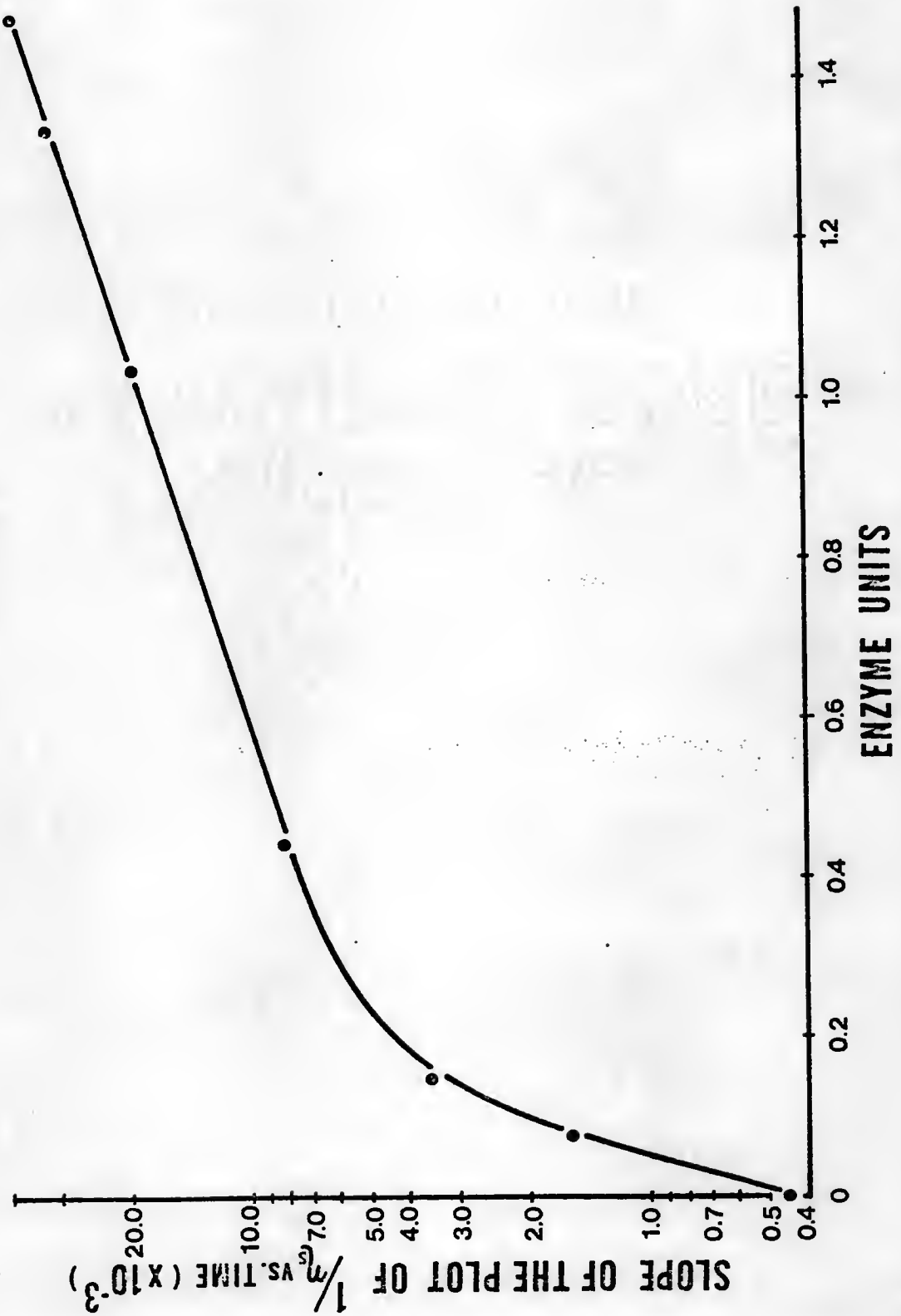
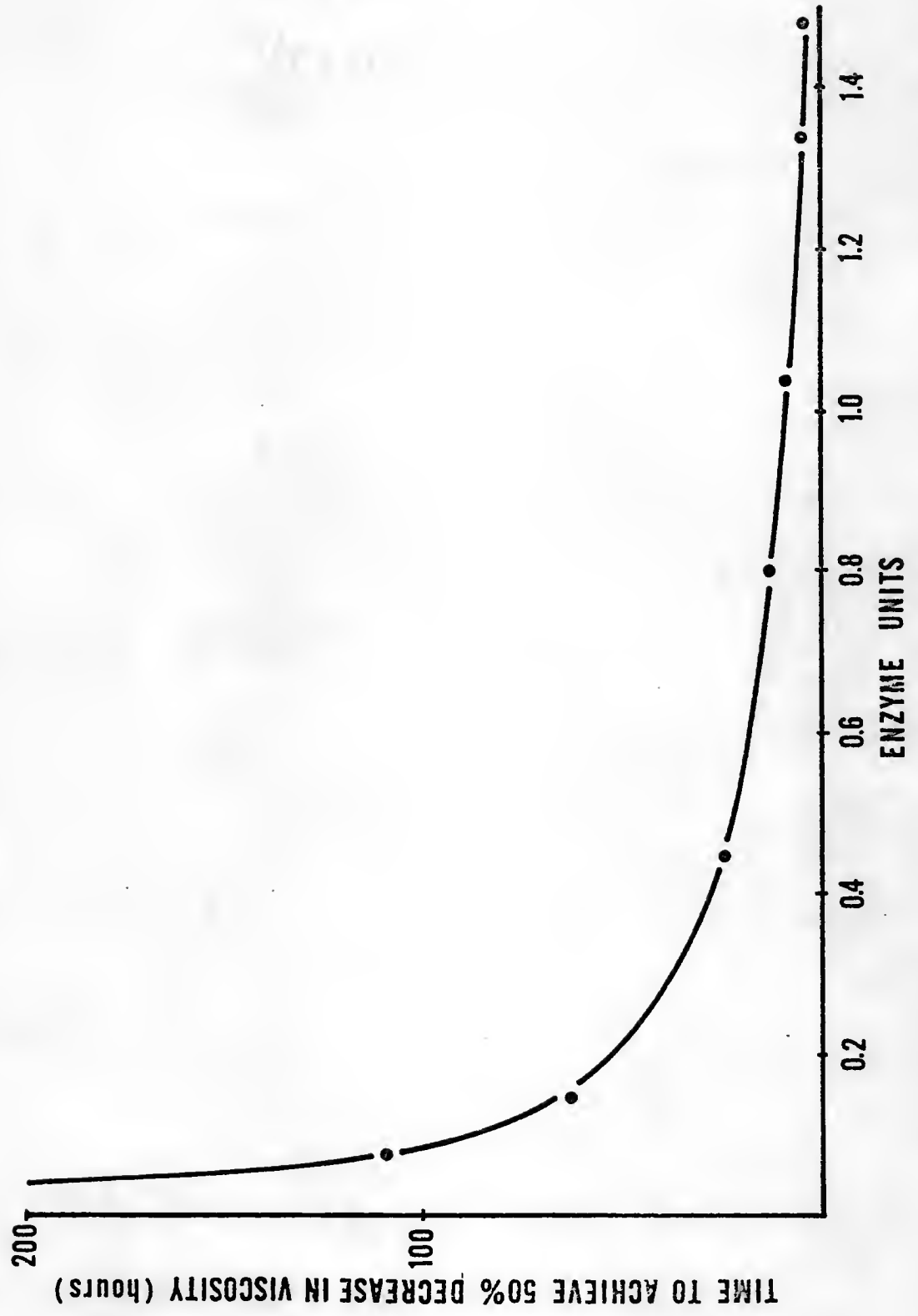

SLOPE OF THE PLOT OF 1/m VS. TIME (X 10-3)
20.0
10.0
7.0
5.0
4.0
3.0
2.0
1.0
0.7
0.5
0.4
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
ENZYME UNITS

FIGURE 14: Relationship between enzyme amount and time to achieve a 50% reduction of viscosity of a CMC solution. The times were calculated from the data shown in Figure 13. Each point is the average of 2 replications.


TIME TO ACHIEVE 50% DECREASE IN VISCOSITY (hours)
200
100
0.2
0.4
0.6
0.8
1.0
1.2
1.4
ENZYME UNITS

APPENDIX II
GROWTH OF *Rhizobium trifolii* IN INO MEDIUM

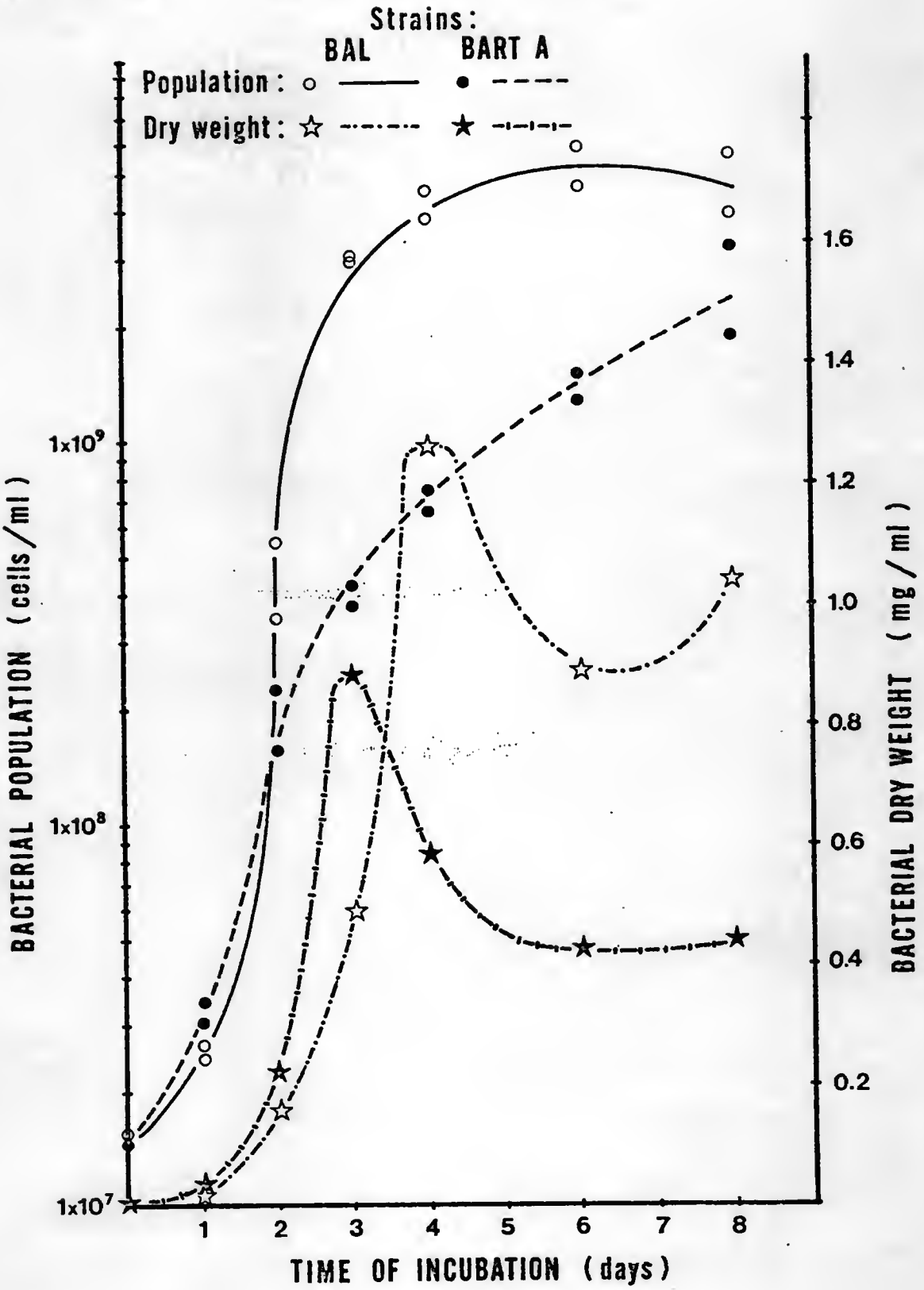
Rhizobium trifolii cultures growing in INO medium produce a dark yellow pigment which strongly absorbs at wavelengths shorter than 400 nm and gives a positive reaction with the Lowry assay for proteins (Lowry et al., 1951). This makes it difficult to quantify protein or bacterial population by UV absorption or optical density. For this reason, bacterial growth was measured by dry weight of bacteria per unit of volume of culture.

Clumping often occurs in *Rhizobium trifolii* cultures in INO medium. For this reason, bacterial population was determined by direct counting using a Petroff-Hauser chamber after 30 sec of sonication at 50W (Heat systems-Ultrasonics Inc., model W140). The dry weight of bacteria was estimated filtering a sample of culture through a Nucleopore filter (Polycarbonate, 0.4 μm, Nucleopore Corp., Pleasanton, CA). The filtrate was washed twice with filtered deionized water and dried overnight at 70°C. The weight of the bacteria was calculated as the difference in weight before and after filtration.

Figure 15 depicts the growth of *Rhizobium trifolii* strains BAL and BART A in INO medium. The dry weights of

bacteria reach a peak toward the end of the log phase of growth and then drop. The reason may be cell lysis and indeed the viable cell count drops during stationary phase. The optical density of the culture at 620 nm continues increasing, however, due probably to accumulation of dead cells, extracellular polysaccharide and pigment. The yellow pigment is produced by Rhizobium trifolii growing in a defined medium with myo-inositol as C source. Yeast extract inhibits pigment production.

FIGURE 15: Growth and dry matter accumulation in Rhizobium trifolii strains BAL and BART A cultures in INO medium. The experiment was performed using duplicate flasks. Each bacterial population point is the average of 4 readings for each flask at each sampling time. Each dry weight point is the average of 4 estimations (2 per flask) at each sampling time.


Strains:
BAL
BART A
Population :
Dry weight :
BACTERIAL POPULATION (cells / ml)
1x10⁹
1x10⁸
1x10⁷
BACTERIAL DRY WEIGHT (mg / ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
TIME OF INCUBATION (days)

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BIOGRAPHICAL SKETCH

Victòr Manuel Morales was born March 3, 1949, in Itsmina, Colombia. He graduated from Colegio de Santa Librada in Cali, Colombia, on July 1965. In December, 1971, he received his degree of Ingeniero Agronomo from the Universidad Nacional de Colombia in Palmira.

He held the position of Research Assistant in CIAT (Centro Internacional de Agricultura Tropical) from January 1972 to March 1975.

Upon being awarded a scholarship from CIAT, he came to the United States for graduate studies. He received his Master of Science degree from the University of Florida in August 1977.

He is a member of the American Society for Microbiology and the Florida Soil and Crop Science Society.

He is currently a candidate for the Ph.D. degree in the Soil Science Department of the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Hubbell, Chairman
Professor of Soil Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward M. Hoffmann
Professor of Microbiology and
Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Chesley B. Hall
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Bob G. Volk
Associate Professor of Soil Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James F. Preston
Associate Professor of Microbiology
and Cell Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1981

Dean, College of Agriculture

Dean for Graduate Studies and
Research

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